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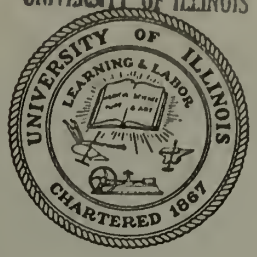

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ROLE OF ENZYMES IN CONTACT STABILIZATION PROCESS

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THESIS

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ROLE OF ENZYMES IN CONTACT STABILIZATION PROCESS

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Department of Civil Engineering

University of Illinois, 1965

The role of enzymes in the removal of organic matter and their activity in the different phases of a contact stabilization process was investigated. There has been much speculation about the mechanism of the primary removal of substrate in the contact tank. Various BOD removal relationships have been formulated in an attempt to explain the mechanism in practice. All these expressions show a direct relationship between the substrate removal capacity of the sludge and substrate concentration. This suggests a physical phenomenon such as adsorption or diffusion. It was shown that the primary mechanism for the removal of soluble substrates is an enzymatic reaction. The rate of substrate uptake can be saturated by increasing the food to microorganism ratio. The kinetics of uptake was found to follow an enzymatic model based on pure culture studies. Temperature was shown to affect the primary removal mechanism in a manner typical of biological reactions.

The activity of different enzyme systems during stabilization was also investigated. It was shown that stabilization of sludge results in a loss of inducible enzyme systems which lowers the substrate removal capacity of the organisms. In batch experiments, the initial increase in the substrate removal capacity of freshly fed sludge during stabilization was attributed mainly to synthesis of enzymes. The subsequent decrease in the activity of sludge, during stabilization, was accounted for by the decrease

in the activity of the rate limiting inducible enzyme system.

In a simulated contact stabilization unit, it was shown that the sludge substrate removal capacity could be increased by decreasing the stabilization period. This observation again substantiated the belief that stabilization impairs the capacity of the sludge organisms to remove substrate. It was also found that long stabilization periods resulted in the release of nondegradable soluble COD in the system. From this study it was concluded that criteria for the time of stabilization should be based upon the settling quality of the sludge, as high loading rates and short stabilization periods may result in bulking sludge.

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I. INTRODUCTION

Many of the unit processes presently used in the treatment of waste water have evolved more from practical experience than from fundamental principles. In considering the treatment of wastewaters by biological methods, a knowledge of the fundamental phenomena which describe the microbiological removal of organic matter from solution would permit greater exploitation of the full potentialities of the unit processes. Current research in the area of biological treatment of wastes has led to the development of more rational design procedures. The activated sludge process, one of the major biological treatment processes, has been studied in great detail. The term "activated sludge" is no longer unexplainable. The sludge consists primarily of a living biological mass in which bacteria form the predominant group. Its behavior can be explained in terms of the activity of these organisms.

The principle of waste treatment by the activated sludge process, like any other biological treatment method, consists of two general reactions, namely, synthesis of cellular material resulting in new cells and respiration. The latter reaction yields energy required for synthesis and maintenance of the sludge organisms. These reactions, though sufficient to explain the gross activity of the system, do not explain many details of the process. Microbiologists using pure cultures and simple substrates have been able to explain many of the laws of growth, substrate removal, enzyme formation, etc., in great detail. The complex nature of the substrate and heterogeniety of the biological population of the system with which the sanitary engineer must necessarily work, makes the elucidation of the laws governing biological waste treatment processes much more

complicated. The concepts developed in sanitary engineering are not always in agreement with those developed from pure culture studies. However, a comparison between a biological waste treatment process and its idealized counterpart involving pure cultures and simple substrates is an essential prerequisite to the evaluation of the fundamental variables responsible for the observed behavior of the complex system.

The research reported herein is concerned with the application of the concepts developed in pure culture studies to activated sludge systems. In particular, the role of enzymes in the removal of organic matter in the contact stabilization process has been investigated.

Studies were also conducted to investigate the effect of different loading rates on the performance of the contact stabilization process and on the biochemical characteristics of the sludge organisms.

The laboratory activated sludge systems were developed on pure organic substrates. It is believed that the information obtained from this investigation, though it may not be quantitatively applicable to the waste treatment process treating complex wastes, will contribute fundamental knowledge regarding the biochemistry of biological systems having a heterogeneous population.

II. LITERATURE REVIEW

A. Historical Background

The activated sludge process, in its conventional form, has been used successfully for the treatment of waste waters for many years. In an attempt to improve the efficiency and application of this biological process and, at the same time, to increase the rate of treatment of wastes, many modifications of the conventional activated sludge process have been proposed and used. In the conventional activated sludge process, an initial rapid removal of organic matter has been observed by many investigators. This has led to the modification of the process known as the contact stabilization process. In this process waste is aerated with stabilized sludge for a short contact period. The sludge is then separated by sedimentation. The settled sludge is transferred to a sludge stabilization unit where aeration is continued to complete the oxidation of stored organic material and to prepare the sludge for BOD removal of fresh incoming waste. The process is based upon the fact that two distinct stages exist in the oxidative metabolism of organic matter by microorganisms: (i) a rapid oxidative conversion of exogenous organic matter into intracellular storage material and (ii) subsequent utilization of stored matter for energy and synthesis of protoplasm (1). Use of this modification permits a reduction in aeration tank capacity. In the conventional process the entire volume of the mixed liquor is aerated through the stabilization stage, whereas in contact stabilization, aeration after the contact period is confined to the return sludge volume only.

The contact stabilization process is also known as "biosorption," "bioflocculation," and "sludge reaeration" (2). The sludge reaeration

process has been in use for a long time. The first full scale activated sludge treatment plant constructed in the United States in 1917 employed sludge reaeration (3). The difference between the early designs and the present day modifications lies in the time periods allowed for contact and stabilization. The old designs generally allotted longer periods of contact, approaching the detention time in a conventional activated sludge process, and shorter sludge reaeration periods. This did not result in any saving of tank volume. In 1938 Mohlman (4) stated that no convincing evidence was ever presented to show that reaeration was either more effective or more economical than a tank of equal capacity devoted to the conventional activated sludge process. In his opinion the most valid reason for the use of the sludge reaeration process was the buffering capacity of the process against poisonous effects of an influx of toxic wastes. Later on other modifications using sludge reaeration were developed by Gould (5) Hatfield (6), and Kraus (7).

The contact stabilization process in its present form was developed by Ullrich and Smith (8)(9) and Zablatsky et al. (10). The process has found general application. Grich (11) has summarized the operational data of a number of such plants. The process has been successfully applied to the treatment of industrial wastes also (11)(12)(13). The advantages of the process over conventional activated sludge have been summarized as follows (2)(3)(11):

(i) Separation of the two stages of substrate removal and oxidation-synthesis in the contact stabilization process, which occur in the same unit in conventional practice, into different units affords greater flexibility in the control of the process since each is then independently controlled.

(ii) It is more capable of handling shock loadings by virtue of the

reservoir of sludge contained in the sludge reaeration unit. In conventional practice a shock load is continuously mixed with the entire return from the final clarifier. In the sludge reaeration system, however, the effects of this shock load are only felt in the mixed liquor aerator which is constantly being fed by a supply of non affected sludge from the reaeration unit.

(iii) Whereas 35 lb of BOD per day per 1,000 cu ft of aeration tank volume is generally accepted as the maximum permissible average loading for conventional plants, reaeration plants have operated at average loadings in excess of three times that figure without sacrifice of effluent quality. This results in smaller tank capacities, less area, and a lower capital expenditure.

(iv) At conventional treatment plants, where sludge bulking is a problem, adoption of sludge reaeration results in more efficient operation and a lower sludge volume index.

(v) Higher BOD removals per unit weight of sludge are conceivable as enzymes produced during the stabilization of mixed liquor in the conventional process may be sufficiently water soluble to be lost in the plant effluent, while enzymes produced during the stabilization of return sludge in a contact stabilization process are reacted with the waste.

Weston and Eckenfelder (14) have pointed out that the initial removal of BOD is of considerable economic importance in waste treatment. It is important because it provides the basis of design for an economical and versatile treatment unit. The mechanism of initial removal and factors influencing it have been the subject of a number of investigations. Some of these are reviewed in the following section.

B. Substrate Removal Relationships in the Contact Stabilization Process

The initial removal of organic matter in the activated sludge process has been observed by many investigators. In these studies, which are reviewed here, attempts have been made to elucidate the mechanism of the initial removal, also known as the first stage or clarification stage of the activated sludge process, and to formulate the kinetics of the process. In order to study the kinetics, a consideration of the mechanism appears to be necessary. However, most of the studies have been directed towards establishing empirical relationships between substrate removed and the sludge and substrate concentrations. This is understandably so, as the design engineer is always interested in such formulations, however crude, to provide a basis for design.

The first step in the metabolism of a substrate, uniformly dispersed in a medium, is the establishment of a concentration gradient in the direction toward the metabolizing organism. Such a gradient can be the result of: (i) a physical adsorption process or (ii) an enzymatic process of permeation into the cell. Early studies (15)(16) on the contact phase of the activated sludge process indicated that only the fraction of total BOD exerted by colloidal or suspended organic matter was removed during this stage. Ingols (17) concluded that the removal of soluble organic substances was brought about by biological or biochemical processes and was independent of purely physical phenomena. Engelbrecht and McKinney (18), while studying activated sludge cultures developed on glucose, suggested that glucose was absorbed by the organisms and subsequently metabolized when all of the glucose was removed from solution. In later studies Gaudy and Engelbrecht (19) concluded that adsorption did not account for the initial removal of soluble organic substrates. Smallwood (20), using

metabolic poisons in activated sludge systems, showed that the significant and controlling mechanism in the removal of soluble organic substrates from sewage by the activated sludge process was assimilation and not adsorption. However, in the case of colloidal organic substrates the significant removal mechanism was adsorption. Woods and Malina (21) while studying the uptake of glycine by anaerobic sludge organisms showed that the initial uptake consisted of a rapid nonbiological uptake which was complete in about 2 min. This was followed by a biological uptake. Carlson and Polkowski (22) have discussed the different possible modes of entry of amino acids into a bacterial cell. However, they did not draw any conclusions.

The kinetics of initial removal have been mostly described by adsorption isotherms. Ruchhoft et al. (23)(24) found that removal of glucose from solution followed a Freundlich isotherm when it was metabolized by activated sludge. Their data did not show an equilibrium stage between the substrate remaining in solution and the fraction removed. Therefore, conclusions based upon the isotherm are questionable. Katz and Rohlich (25) observed such an equilibrium and suggested that a modified form of Freundlich isotherm characterized their data. They did not preclude enzymatic action in the removal of substrate in their experiments.

Gellman and Heukelekian (26) demonstrated that the rate of substrate removal in the first stage of the activated sludge process was higher than the rate of oxygen uptake. Similar findings were reported by Porges et al. (27). Working with skim milk they found the rate of purification, or substrate removal, was four times the rate of oxygen uptake. Oxygen uptake continued at the same rate for an appreciable length of time even when all the skim milk had been removed from solution. From this

they concluded that there was a nonoxidative accumulation of organic matter in the organisms which underwent subsequent rapid oxidation.

Weston and Eckenfelder (14), with reference to the studies by Porges et al. (27) and Hoover et al. (28), postulated that the enzyme responsible for the initial removal reacted with the waste substrate to form an organic matter-enzyme complex. This complex was stored by the cell as a source of energy and material for cell growth for future use. On the basis of this postulation they developed the following equation:

$$L_{ri} = \frac{K_1}{K_2} \cdot \frac{e}{p} \cdot L_o S_o$$

or

$$L_{ri} = b \cdot L_o S_o \qquad 1$$

where L_{ri} is the BOD removed initially in a given contact period, L_o and S_o are the initial concentrations of BOD and sludge respectively, e is the concentration of enzyme per unit weight of sludge, K_1 and K_2 are rate constants, p is a proportionality constant, and b is equal to $\frac{K_1}{K_2} \cdot \frac{e}{p}$.

Equation 1 was reformulated by Eckenfelder (29) as follows:

$$\frac{-dL}{dS_o} = K_i L$$

which integrates to:

$$\frac{L_{ri}}{L_o} = (1 - e^{-K_i S_o}) \qquad 2$$

where K_i is the constant for initial removal rate.

The effect of stabilization of sludge on its activity has been recognized. Keefer and Meisel (30) reported an increase in the substrate removal capacity of sludge with reaeration. Similar results were reported by Eckenfelder (29). However, he also showed a decrease in sludge activity with prolonged stabilization periods. Results on the rate of removal of lactose by activated sludge reported by Wuhrmann (31) indicated a similar decrease in the removal rate with increasing stabilization periods. These observations were also substantiated by Lenhard et al. (32).

Although the mathematical expressions (Equations 1 and 2) described above appear to conform to the observed behavior of the contact stabilization process, they have significant limitations. Enzyme substrate complex in an enzyme catalyzed reaction is of an extremely transient nature. Direct experimental evidence for the existence of such compounds has been difficult to obtain (33). The idea of an energy rich enzyme substrate complex which can be stored by a cell for future use, as postulated by Eckenfelder (14), is without basis and fact. The initial removal of BOD without a proportionate increase in oxygen uptake, and the initial increase of biological solids, can be amply explained, as pointed out by Wilson (34), by the fact that the many consecutive reactions in the metabolism of a substrate require concentrations of intermediate products to drive the various reversible enzyme reactions towards completion. Furthermore, Equations 1 and 2 will not hold for high food to organism ratios when the limiting enzyme becomes saturated by the substrate. A further increase in substrate concentration over this level is without effect on the rate of uptake since the limiting enzyme reaction rate is now completely saturated (33).

C. Bulking of Sludge

The successful operation of the contact stabilization process depends upon striking a balance between the following considerations:

(i) Initial increase in the removal rate of substrate with increasing stabilization periods.

(ii) Decrease in the activity of sludge with prolonged stabilization periods.

(iii) Bulking of sludge with high loading rates or short stabilization periods.

Because of the short contact periods, a contact stabilization process approaches the conditions of a chemostat with decreasing stabilization periods. Herbert et al. (35) while culturing Aerobacter cloacae in a chemostat showed that an increase in the dilution rate (ml of substrate/hr/ml of reactor volume) shifted the generation time of the organisms to a lower value proportional to the reciprocal of the dilution rate. There was no increase in the concentration of the substrate in the effluent with increasing dilution rates until the reciprocal of the dilution rate resulted in the minimum generation time of the organism. Above this dilution rate the organisms were washed out of the system and the substrate concentration in the effluent increased sharply. This study indicates that the removal of soluble COD in a contact stabilization process will not decrease with short stabilization periods, provided the organisms can be returned to the system. Therefore the controlling factor in the selection of operating parameters appears to be the settling quality of the sludge. A good settling sludge can be separated in the sedimentation tank and returned to the system.

Overloading of an activated sludge system has been cited as the

cause of bulking. A loading rate of 0.5 lb of BOD/day/lb of mixed liquor volatile suspended solids has been recommended for a design criterion for activated sludge systems (36). Orford et al. (37) showed a log relationship between sludge volume index and loading rate. From pilot plant studies Logan and Budd (38) reported a minimum sludge volume index at a loading rate of 0.3 lb of BOD/day/lb of mixed liquor volatile suspended solids and higher values for both higher and lower loading rates. Contrary to these findings, Stewart (39) reported a maximum sludge volume index at a loading rate of 0.6 lb of BOD/day/lb of mixed liquor volatile suspended solids and a lower sludge volume index for both higher and lower loading rates.

It should be noted that bulking of sludge may result from two different causes, a Sphaerotilus growth and a dispersed growth. Ingols and Heukelekian (40) and Gentelli and Heukelekian (41) attributed bulking to the growth of Sphaerotilus. They also reported that the chance of these organisms predominating was greater when the system was maintained on carbohydrate substrates. McKinney (42) stated that biological flocculation as it occurs in a biological waste treatment process is not the result of any special zoogloea-producing bacteria, but is the result of environmental conditions. Actively multiplying organisms did not settle and remained in a dispersed state.

By decreasing the stabilization time in a contact stabilization process, therefore, bulking due to a dispersed growth will be induced. Hence the minimum stabilization time will be governed by the flocculating properties of the organisms.

D. Pure Culture Studies, Bacterial Permeases

Fermentation of sugars by yeasts has provided several of the

earliest puzzling phenomena of selective permeation or crypticity. Cryptcity is defined as the impermeability of a substrate into a cell in the presence of intracellular enzymes active against the substrate, i.e., the enzymes are hidden from the substrate. Leibowitz and Hestrin (43) reported instances where organisms capable of fermenting monosaccharides and disaccharides were cryptic towards certain other disaccharides even though they were shown to contain the necessary hydrolyzing enzymes. Similarly Deere et al. (44) described a mutant of Escherichia coli which synthesized lactase constitutively but the intact cells did not show any lactase activity unless they were acclimatized to lactose. Doudoroff et al. (45) reported a striking example of crypticity where a mutant strain of E. coli utilized maltose but did not use glucose, a monomer of the disaccharide. Karlson and Barker (46) reported that Azotobacter agilis utilized acetate constitutively via the Kreb's cycle, while the intermediates of the cycle were not metabolized. Stone and Wilson (47) showed that these intermediates were oxidized by cell free extracts. Barett et al. (48) reported that cell extracts of Pseudomonas flourescens, grown on fumarate, oxidized citrate. However, for in vivo oxidation of citrate the cells required a period of acclimation.

The circumstantial evidence provided by the above studies for the existence of a selective transport system in bacteria and other studies, to be described later, led Cohen and Monod (49) and Rickenberg et al. (50) to postulate a mechanism of active transport of substrates into cells. They designated the proteins involved in the transport by the generic name "permeases." Permeases are defined as stereospecific systems, which mediate an active transport of nutrients into a cell and which are functionally specialized and distinct from metabolic enzymes.

The kinetics of removal of substrate from solution by bacteria has been formulated mostly by studying the uptake of thiomethyl-β-D-galactoside (TMG) by E. coli (49). This analog of the β-galactosides in which the oxygen atom of the glycosidic linkage is substituted by sulfur is not hydrolyzed by galactosidase, nor is it metabolized by E. coli. When a suspension of E. coli, previously induced by growth in the presence of a galactoside, is shaken with TMG, the cells accumulate the analog. Rickenberg et al. (50) showed that the accumulation was reversible. By allowing the cells to attain equilibrium with radioactive species of TMG and then adding an excess of nonradioactive TMG, the accumulated radioactivity of the cells could be diluted out. They described the concentration of intracellular substrate at equilibrium according to a Langmiur adsorption isotherm expressed as:

$$G_{in}^{eq} = Y \frac{G_{ex}}{K_m + G_{ex}} \qquad 3$$

where G_{in}^{eq} is the intracellular substrate concentration at equilibrium, G_{ex} is the initial extracellular substrate concentration, K_m is an apparent dissociation constant, and Y is the specific capacity, i.e., the maximum amount the cell can accumulate. Kepes (51) further extended these studies and showed that the initial rate of uptake may be expressed by the following equation:

$$\frac{dG_{in}}{dt} = V_{in}^{max} \frac{G_{ex}}{K_m + G_{ex}} - k_{ex} G_{in} \qquad 4$$

where G_{in} is the intracellular substrate concentration at any time, V_{in}^{max} is

the maximum velocity of uptake, and k_{ex} is the exit rate constant. Equation 4 is the Michaelis law, since it applies to the velocity of a reaction. K_m of this equation is the same as that of Equation 3. Equation 4 upon integration yields:

$$G_{in} = \frac{V_{in}^{max}}{k_{ex}} \cdot \frac{G_{ex}}{K_m + G_{ex}} (1 - e^{-k_{ex}t}) \qquad 5$$

For steady state conditions, when the rate of entry of substrate is equal to the rate of exit, Equation 4 can be rewritten as:

$$G_{in}^{eq} = \frac{V_{in}^{max}}{k_{ex}} \cdot \frac{G_{ex}}{K_m + G_{ex}} \qquad 6$$

Comparing Equations 3 and 6 and 5 and 6, the following relations were obtained (50):

$$Y = \frac{V_{in}^{max}}{k_{ex}} \qquad 7$$

and

$$\frac{G_{in}^{eq} - G_{in}}{G_{in}^{eq}} = e^{-k_{ex}t} \qquad 8$$

Therefore, according to Equation 8, the exit rate constant, k_{ex}, is given by the slope of the straight line obtained from a plot of log $(G_{in}^{eq} - G_{in})$ versus time.

The observations which led to the conclusion that the initial rate of removal, as described by Equation 4, is catalyzed by an enzyme are summarized below:

(i) Specific mutations inactivated the ability of the organisms

to accumulate substrates (50).

(ii) In organisms which had to be induced in order to be able to accumulate substrate, the process of induction followed the pattern of a typical synthesis of an inducible enzyme (50).

(iii) The rate of uptake, as given by Equation 4, could be saturated at high substrate concentrations (52).

(iv) The exit rate was shown to be a crrier mediated process rather than free diffusion (51).

Like the analogs of β-galactosides, α-methyl glucoside has been reported to be accumulated by suspensions of E. coli (53). The values of K_m in the Equation 4, for two different strains of E. coli, were reported to be 2×10^{-4} M (53) and 5×10^{-5} M (54). Studies with E. coli and Salmonella typhimurium showed that mutations which resulted in the loss of the α-methyl glucoside permease system also impaired the ability of the organisms to metabolize glucose (53)(54)(55). From these studies it was concluded that α-methyl glucoside and glucose shared the same permeation system (53)(55).

The expenditure of energy during the transport of the substrate into the cell was studied by Kepes (56). On the basis of oxygen uptake studies he concluded that one high energy phosphate bond was utilized per molecule of thiogalactoside transported by an E. coli permease system.

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III. SCOPE OF INVESTIGATION

As mentioned previously, bacteria can be regarded as the predominant group of organisms constituting activated sludge, both structurally and functionally. The reactions of oxidation and synthesis, fundamental to an activated sludge system, are performed by these organisms through the aid of enzymes. Enzymes, in addition to acting as catalysts, bring about a controlled stepwise decomposition of organic matter and synthesis of new cellular constituents. They also make available to the cell a portion of energy resulting from respiration by synthesizing energy rich compounds.

A bacterial cell can be schematically represented as indicated in Figure 1. The intracellular enzyme systems can be divided into two broad categories depending upon their functions: hydrolases and synthesizing and respiratory enzymes. Another set of enzymes, whose presence in bacterial cells has been demonstrated, are the permeases. They mediate the transport of exogenous substrate into the cell. Once the substrate is inside, the hydrolases break it down in order to make it available for further reactions. This is not an obligatory step and is by-passed if the substrate molecule is in a form consistent with the scheme of reactions catalyzed by the synthesizing and respiratory enzymes. This last system includes the enzymes associated with the major pathways of respiration and synthesis.

Not included in the scheme of enzyme systems outlined above are the extracellular hydrolases which are secreted by the cells to hydrolyze long polyomeric substrates into smaller units. This partially hydrolyzed

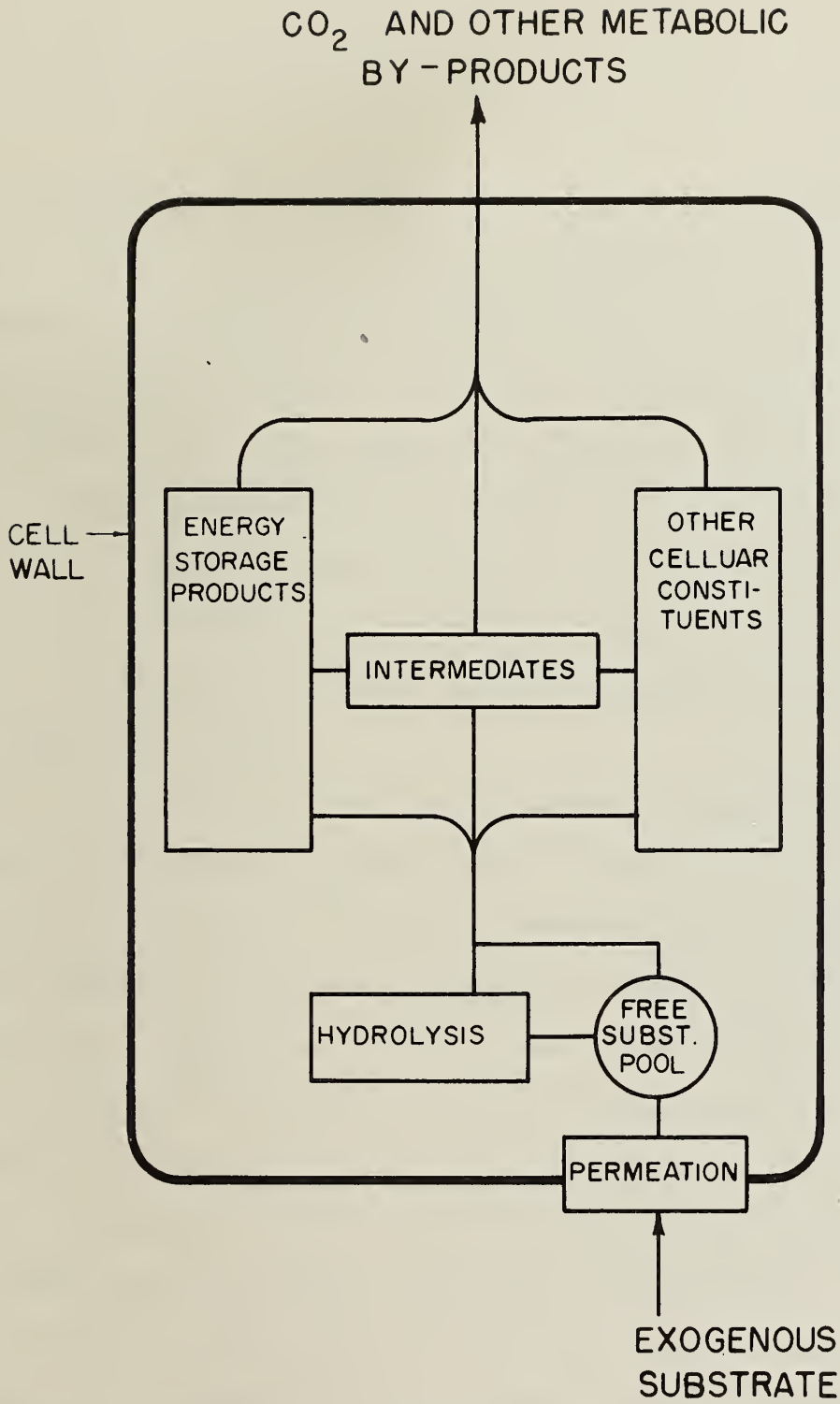


FIGURE I. SCHEMATIC REPRESENTATION OF A BACTERIAL CELL AND ITS BIO-CHEMICAL ACTIVITIES

substrate is then transported into the cells from the medium. Usually these compounds are further hydrolyzed by the specific intracellular hydrolases.

The enzyme systems described above may not be present in a cell at all times. The cell may have the ability to synthesize all the specific enzymes necessary for utilizing a particular substrate, but may require an inducer to initiate their synthesis. In general, substrates serve the function of inducers also. These enzymes which appear in the cell in response to a stimulus from outside and disappear when the stimulus is removed are called inducible enzymes. However, all living cells are endowed with certain enzymes which are constantly present in all cells of that species. Such enzymes, which are a normal constituent of the cell, are called constitutive enzymes.

In a conventional activated sludge system the sludge organisms are in contact with the waste or substrate for a period of six to eight hours. This period of time is sufficiently long on the physiological time scale of the organisms to permit the organisms to synthesize inducible enzymes which may be required for the utilization of the particular waste. In the contact stabilization process, on the other hand, the sludge is in contact with the waste for only a short period of time, usually one-half to one hour. The sludge is then separated from the waste and returned to the stabilization tank for endogenous oxidation of stored material. Therefore, for the system to operate satisfactorily, the sludge organisms should have a complete set of preformed enzyme systems. It should be noted that, during the period of sludge stabilization, organisms could lose the activity of those inducible enzymes which are directly responsible for the initial removal of organic matter in the contact tank. This suggests a

direct relationship between the system efficiency and the level of different enzymes in the organisms.

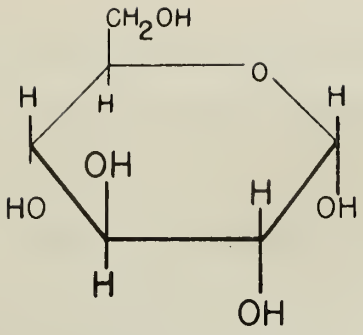
The present study was carried out to relate the behavior of the contact stabilization process to the activity of different enzyme systems. As reviewed in the previous chapter, there is a controversy in sanitary engineering practice regarding the existence of the permease systems. Therefore, experiments designed to differentiate between enzymatic transport and physical adsorption of the substrate were conducted. An attempt was also made to optimize the duration of the stabilization period with respect to the performance of a contact stabilization unit.

IV. MATERIALS AND METHODS

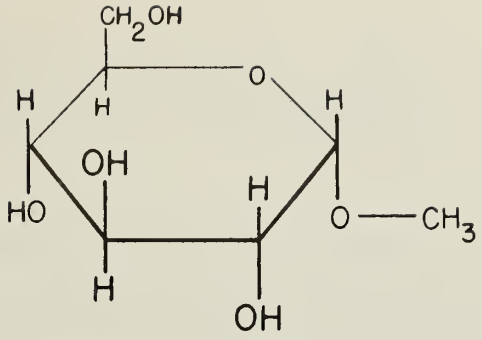
A. Experimental Design

1. Studies of initial substrate uptake

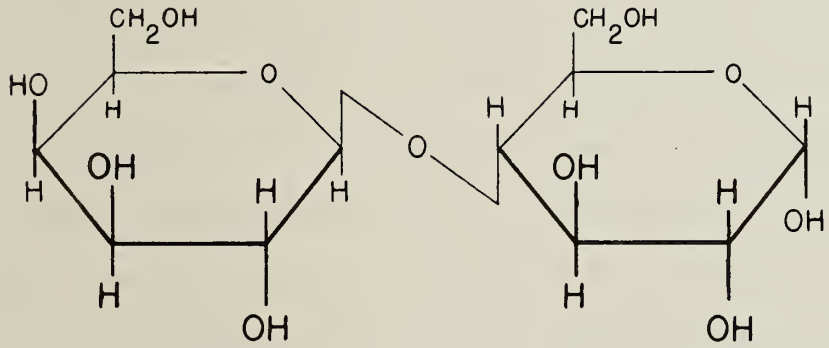
The kinetics associated with immediate uptake of organic material by sludge organisms has been formulated in sanitary engineering research primarily on the basis of complex substrates. The complexity of the substrate introduces a number of reaction rates, of different characteristics, which make if difficult to analyze and interpret the results. This is particularly true when the substrate consists of both suspended and dissolved organic matter. If it is assumed that the dissolved organic matter is removed by enzymatic reaction and the suspended by a physical adsorption process, the resulting kinetics of removal of organic matter, as measured by the BOD test, will be governed by the relative concentrations of the two forms of substrates. For this reason, soluble organic compounds were used as substrates in this study. The structures of the substrates are shown in Figure 2. Glucose and α-methyl glucoside were used to investigate the kinetics of immediate substrate removal. The rate of initial removal of glucose from solution by microorganisms is influenced by the secondary reactions of respiration and synthesis. With pure cultures chloromphenicol has been used with success to control these secondary reactions (57). However, in the present investigation, using a heterogenous population, it was found that chloromphenicol introduced only an initial lag in the metabolism of glucose. It was mentioned in the previous chapter that E. coli does not measurably metabolize α-methyl glucoside, although it does accumulate this compound internally. Further, there is evidence of glucose



GLUCOSE

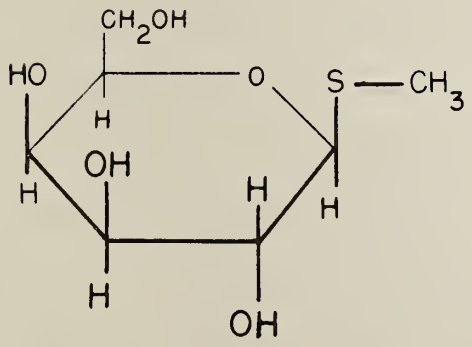


α-METHYL GLUCOSIDE

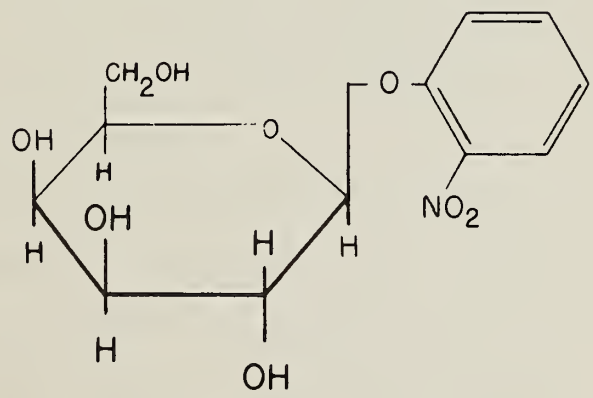


GALACTOSE GLUCOSE

LACTOSE



THIOMETHYL-β-D-GALACTOSIDE
(TMG)



O-NITROPHENYL-β-GALACTOSIDE
(ONPG)

FIGURE 2. SUBSTRATES USED IN STUDY

and α-methyl glucoside being removed by the same permease system. These considerations led to the decision to use glucose and α-methyl glucoside as substrates.

Sludge was obtained from a laboratory fill and draw activated sludge unit described later. In these studies it was necessary to have a readily filterable mixed liquor so as to be able to rapidly separate sludge organisms from substrate in solution. Ten ml of mixed liquor from this unit, having a biological solids concentration of about 1000 mg/ℓ, could be filtered through a 0.45 μ pore size membrane filter disc in less than 10 seconds. To obtain better reproducibility with respect to the physiological state of sludge organisms, a chemostat was also used. However, this source of sludge had to be discarded as it was not possible to rapidly separate the sludge from the substrate by filtration. An attempt to chemically flocculate the bacterial suspension gave erratic results. This was probably due to the leaching out of substrate from the organisms and to the rupturing of cells.

In order to study the mechanism of initial removal of substrate two different approaches were used: (i) to find the saturation level of uptake rate with respect to substrate concentration and (ii) to study the effect of temperature on the rate of substrate removal.

According to the adsorption model, the rate of uptake is directly proportional to the concentration of substrate. Therefore, if this is true, the initial rate cannot be saturated by increasing the substrate concentration. On the other hand, the rate of substrate removal based upon enzymatic transport can be saturated if the substrate concentration is increased to such a level that the transporting enzyme molecules are saturated at all times. Different substrate to microorganism ratios were obtained by varying

the substrate concentration for the same amount of sludge and also by changing the sludge concentration while maintaining a constant substrate concentration.

The rate of an enzyme catalyzed reaction, like a chemical reaction, increases with an increase in temperature. Because of the protein nature of an enzyme, however, thermal denaturation occurs with increasing temperatures and consequently there is a decrease in the rate of activity.

On the other hand, in a physical adsorption process, adsorption has been observed to decrease with an increase in temperature (58). Therefore, observations on rate of initial removal of substrate at different temperatures should substantiate the mechanism of removal.

2. Stabilization of sludge

a. Biochemical changes in organisms during stabilization of sludge

Lactose was used as the substrate for these studies. The choice was made in accordance with the following considerations:

(i) The substrate should be sufficiently complex to require the enzyme systems of permeation, hydrolysis, and respiration and synthesis for its utilization.

(ii) The substrate should be a pure compound measurable by a simple test.

(iii) Analytical methods should be available for assaying the different enzyme systems.

Lactose is a disaccharide (Figure 2). After it is transported into the cell, it is hydrolyzed into its monomers, galactose and glucose, by the enzyme β-galactosidase. Hydrolysis is an obligatory step in the

utilization of lactose. Therefore, organisms which are not synthesizing β-galactosidase cannot use lactose, exceptions being certain members of the genus Pseudomonas which can oxidize lactose to lactobionic acid. However, for the oxidation to proceed any further the acid has to be hydrolyzed to galactose and gluconic acid (59). The enzyme β-galactosidase catalyzes the following general reaction (60):

$$\text{Gal-O-R} + \text{R'OH} \rightleftarrows \text{Gal-O-R'} + \text{ROH}$$

where Gal represents the galactose moiety. For a hydrolytic reaction R' can be replaced by H and in the particular case of lactose, R by a glucose moiety.

The enzyme systems of permeation and synthesis and respiration are necessary for the utilization of any substrate. Therefore, they take part in the metabolism of lactose also.

β-galactosidase was assayed directly by using o-nitrophenyl β-galactoside as a substrate (Figure 2). In this case, R in the general equation given above can be replaced by the o-nitrophenol moiety. When this compound is hydrolyzed, o-nitrophenol is released into solution, imparting a soluble yellow color to the medium. The intensity of color is taken as a measure of enzyme activity. Details of the test are given in the section on analytical techniques.

The other two enzyme systems were measured indirectly. The β-galactosidase permease system was measured by comparing β-galactosidase activity in vivo with the activity in lysed cell preparations. A higher activity of the lysed cell preparation indicated a rate limiting step of transport of substrate into the cell. Therefore, the in vivo activity

measured the activity of the permease system. The activities of the respiratory and synthesizing enzymes, responsible for lactose assimilation, were estimated in terms of the rate of removal of glucose by the organism. Glucose, being a monomer, by-passes the hydrolytic step and takes the same route in the scheme of metabolism as a lactose molecule after hydrolysis. There is a limitation to this reasoning, however, due to the fact that a fall in the rate of removal of glucose may occur due to the inactivation of the glucose transport system and not the enzyme systems of respiration and synthesis.

The final consideration in the choice of substrate is easily met by lactose as it can be measured by the anthrone test.

The sludge for the stabilization experiments was obtained from two sources: (i) a fill and draw laboratory activated sludge unit maintained on lactose and (ii) sludge grown on lactose from a small inocculum and harvested as the organisms entered the stationary phase of growth. This sludge has been designated as "young cell sludge." The essential difference between the two types was that the sludge organisms from the former source were subjected to periods of starvation while those from the latter source were always in contact with substrate. This was done to compare the activity of enzyme systems when sludge organisms were subjected to a process of selection through starvation.

Changes in the enzyme systems during the period of sludge stabilization were studied by aerating the sludge organisms in the absence of substrate. Before stabilization was initiated the sludge was kept in contact with the substrate for 0.5 hour. Samples of sludge were withdrawn at different time intervals and tested for the activities of different enzyme systems. The sludge was also analyzed for its different cellular constituents.

b. Effect of stabilization period on the performance of contact stabilization process

Studies described in the previous two sections attempted to delineate certain fundamental principles involved in the metabolic activity of a heterogeneous microbiological population. This experiment was conducted to study the performance of the contact stabilization process as a whole in terms of substrate removal rate and settleability of sludge under different loading rates.

In studying the biological treatment of waste waters, pure carbohydrates have been used for the most part to develop a heterogeneous population of aerobic organotrophic bacteria. However, it has been reported that most of the sludge bacteria required either preformed vitamins or amino acids, or both (61). Zoogloea ramigera, one of the major organisms reported to be responsible for flocculation in activated sludge, has been shown to require certain growth factors (61)(62). Therefore, with pure carbohydrate substrates, growth of such organisms will be restricted as they will have to depend upon other organisms which synthesize and release their specific requirements. To retain a larger number of species of organisms from activated sludge, a 5:1 mixture of glucose and yeast extract was used as the substrate in these studies. Yeast extract contains most of the amino acids and growth factors ordinarily required by microorganisms.

The loading on the system was gradually increased from 0.8 to 9.6 mg COD/mg sludge solids/day. This was done by increasing the frequency of feeding. The performance of the system, in terms of COD removal rates and settleability of the sludge, was recorded throughout this period. Sludge was also analyzed for protein and carbohydrate content.

B. Analytical Techniques

1. Biological solids

Biological solids were determined by the membrane filter technique. Engelbrecht and McKinney (63) reported a greater precision with the membrane filter technique than with the Gooch crucible method (64). However, Smith and Greenberg (65), in an evaluation of different methods for determining suspended solids, reported that the difference in results was statistically insignificant. In the present investigation the procedure described by Winneberger et al. (66) was used. This method eliminates oven drying which results in a loss of weight of the membrane filter.

Standard filter discs, 47 mm in diameter and having a pore size of 0.45 μ, were used for all solid determinations.

2. Carbohydrates

The concentration of carbohydrates in mixed liquor filtrate was determined by the anthrone test. The test has been in use with different modifications (67)(68)(69)(70). In the present investigation the method outlined by Scott and Melvin (70) was used. The samples were delivered near the bottom of the test tube containing the anthrone reagent by a blow pipette. Good mixing was obtained by blowing air through the pipette. Each analysis of a set of unknown samples included at least three standard carbohydrate solutions. Optical density was measured at a wave length of 620 mμ.*

*All optical density measurements in these studies were made with a "Spectronic 20" spectrophotometer, Bausch and Lomb, Inc., Rochester 2, New York.

3. Chemical oxygen demand, COD

Potassium dichromate in the presence of concentrated sulfuric acid was used as an oxidizing agent. The reaction proceeds according to the following equation:

$$Cr_2O_7^{=} + 14H^{+} + Ge = 2Cr^{+++} + 7H_2O$$

The details of the test are given in Standard Methods for the Examination of Water, Sewage and Industrial Wastes (64). Silver sulfate was used as a catalyst in all determinations. Its use is recommended for more complete oxidation of straight chain compounds; however, it reacts with chlorides, bromides, and iodides to produce precipitates which are only partially oxidized. To overcome the difficulties caused by the presence of chlorides in the sample a modified method was used (71). This method involves a complexing technique for the elimination of chlorides from the reaction. This is accomplished by adding mercuric sulfate to the sample before refluxing. Mercuric sulfate reacts with chloride ion to form soluble mercuric chloride complex which greatly reduces its ability to react further.

The quantities of chemicals suggested in Standard Methods (64) were also reduced. The procedure which was employed is outlined below.

Mercuric sulfate (0.4g) was placed in a 250-ml refluxing flask. A 20-ml sample or an aliquot diluted to 20 ml was added to the flask and swirled to mix. This was followed by 10.0 ml of 0.25 N potassium dichromate solution and 30 ml of concentrated sulfuric acid. The sulfuric acid was prepared by adding 22 g of silver sulfate to a 9-lb bottle of acid. After refluxing for 2 hours the mixture was diluted to 140 ml with

distilled water in the same flask and after cooling titrated with 0.1 N ferrous ammonium sulfate.

4. β-Galactosidase assay

Hydrolysis of o-nitrophenyl β-D-galactoside (ONPG) by sludge organisms was used as a measure of β-galactosidase activity. The method has been described by Lederberg (72). The intact glycoside has a negligible optical density at visible wave lengths. Free o-nitrophenol (ONP), however, gives a yellow color in alkaline solutions. ONP is a weak acid (pH = 7.3) and therefore at pH 10 or higher, only a negligible fraction remains undissociated and colorless. Figure 3 shows the absorbency of ONP in 0.1 M sodium carbonate solution at different wave lengths. It also shows the absorbance of different concentrations of ONP in 0.1 M Na_2CO_3 solution at 415 mμ where the absorption peak occurred.

In the procedure 9 ml of a suitable dilution of sludge suspension was shaken with 1 ml of 0.01 M ONPG by a vortex mixer. After 15 minutes of mixing, 1 ml of a 1.0 M sodium carbonate solution was added to stop the reaction and also to raise the pH of the suspension. Reagent blanks were prepared by adding 1 ml of sodium carbonate to 9 ml of the sludge suspension and 1 ml of distilled water. The alkali was added at the same time to the blank and the assay tube, as it causes a decrease in absorbency of the cells. Optical density was measured at a wavelength of 415 mμ. All readings were corrected for dilution due to the added solutions.

The above procedure was validated by observing the hydrolysis of ONPG by lactose acclimated sludge, as a function of time (Figure 4). The relationship between optical density and concentration of ONP was obtained from Figure 3. The liberation of ONP with different concentrations of

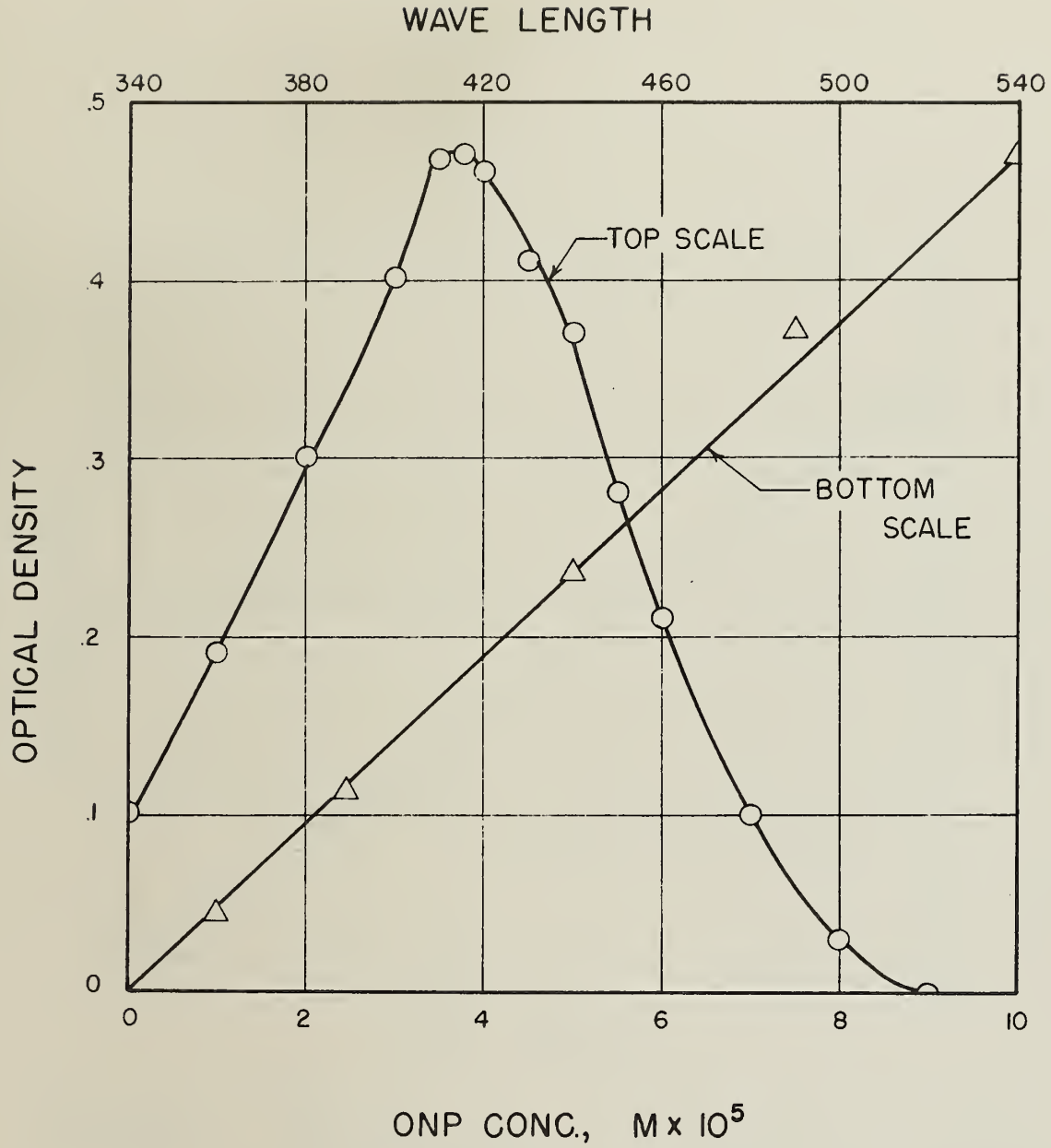


FIGURE 3. OPTICAL DENSITY OF DIFFERENT CONCENTRATIONS OF ONP IN .1M Na_2CO_3 AND OPTICAL DENSITY OF 10^{-4} M ONP IN .1M Na_2CO_3 AT DIFFERENT WAVE LENGTHS

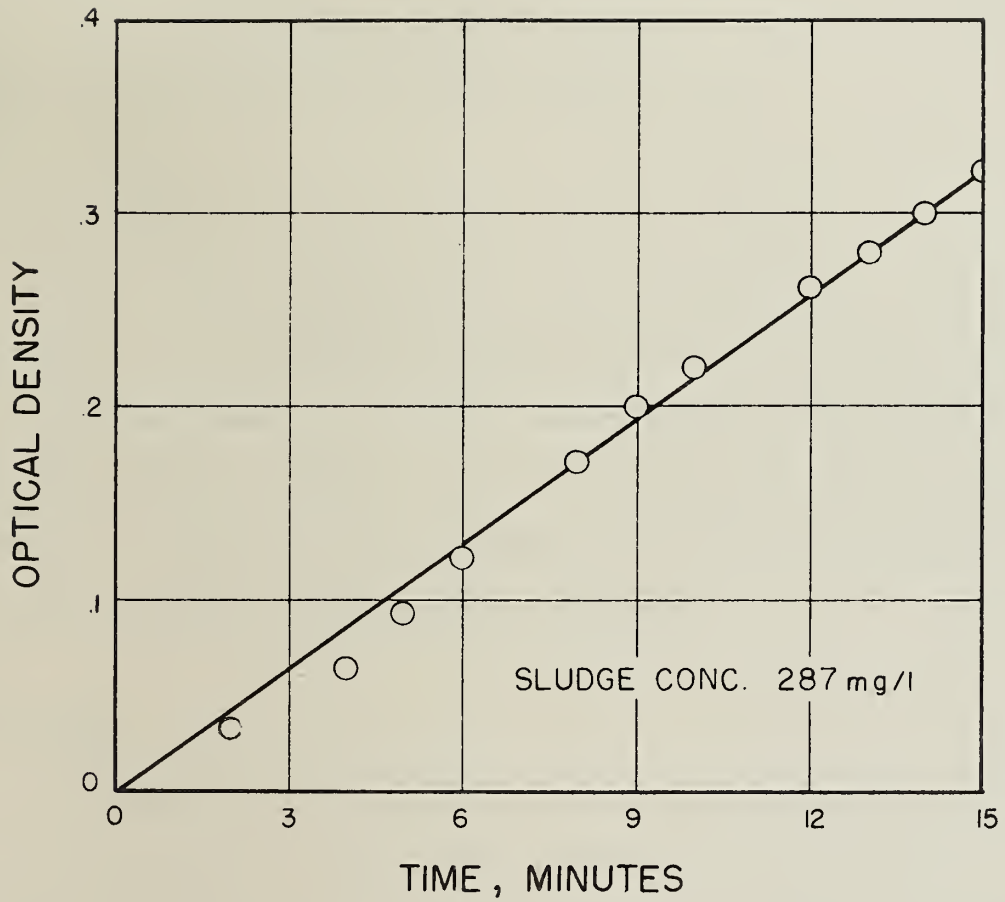


FIGURE 4. TIME COURSE OF ENZYMATIC HYDROLYSIS OF ONPG BY LACTOSE ACCLIMATED SLUDGE

sludge and therefore of enzyme, in 15 min under standard conditions is shown in Figure 5. The linearity of these relationships fulfills the desiderata of an enzyme assay procedure.

The results of β-galactosidase assay have been reported in "activity units." The unit of β-galactasidase activity of a sludge suspension is defined arbitrarily as the liberation of 1×10^{-4} M ONP per 15 minutes, corresponding to an optical density of 0.47.

5. Oxygen uptake

Oxygen uptake was measured by a Warburg Respirometer. The flasks and the manometers were calibrated using the ferricyanide-hydrazine method (73). The concentrations of buffer and minerals used in these studies were the same as shown in Table 1, to which the sludge organisms were acclimated. The experiments were initiated by tipping the substrate in from the side arm, after the flasks had equilibrated.

6. Protein

Cellular protein was measured by the Folin reaction (74). The modified procedure given by Orme-Johnson (75) for the determination of sludge protein was followed. Crystalline bovine serum albumin was used as the standard protein. The standard protein curve shown in Figure 6 was used to determine the protein content of unknown samples.

7. Estimation of β-galactoside permease activity

Rickenberg et al. (50) have measured the activity of permease by the capability of bacterial cells to accumulate thiomethyl-β-D-galactoside (TMG). They showed a straight line relationship between the

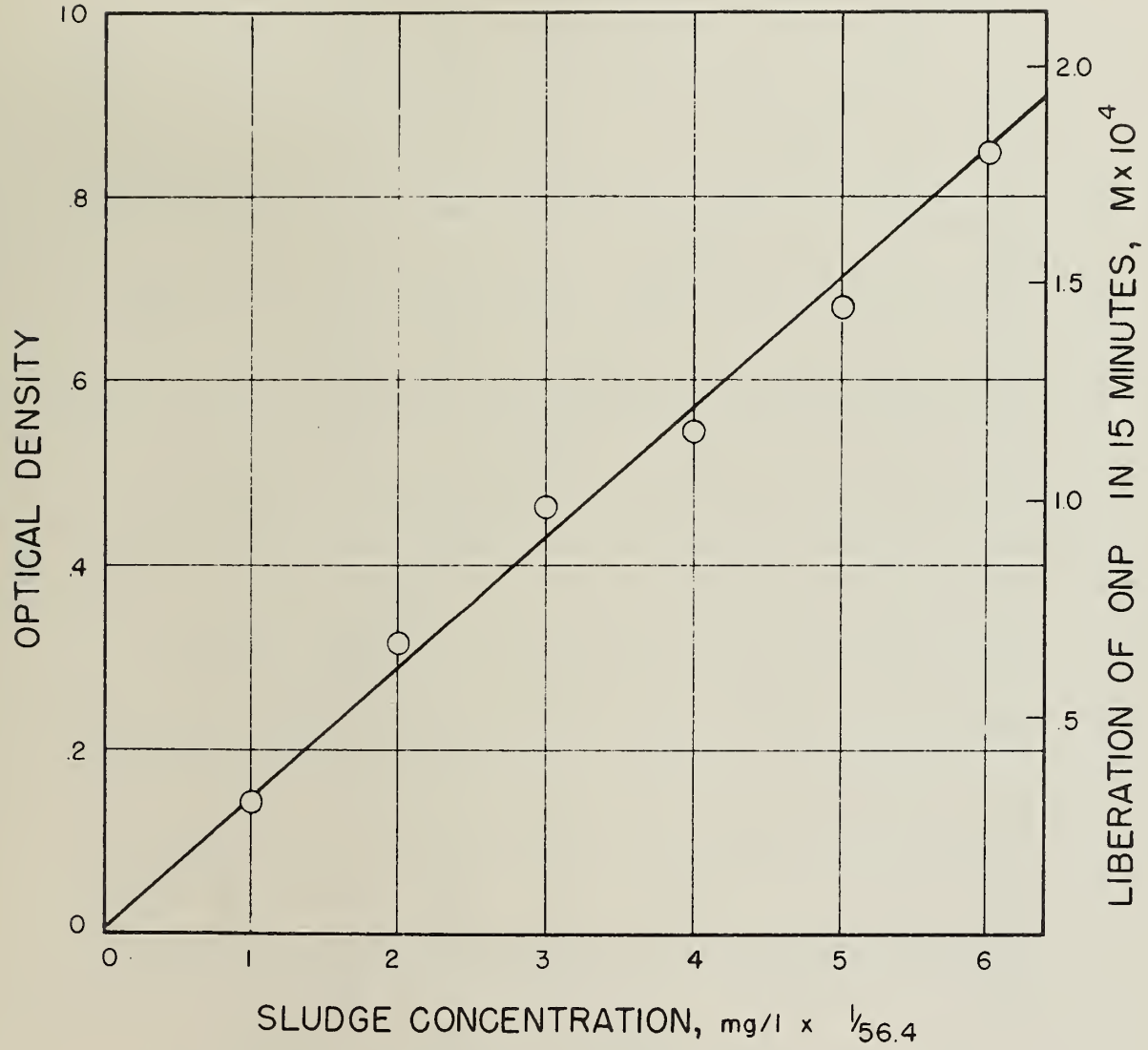


FIGURE 5. ONPG HYDROLYSIS IN 15 MINUTES AS A FUNCTION OF LACTOSE ACCLIMATED SLUDGE CONCENTRATION, UNDER STANDARD CONDITIONS

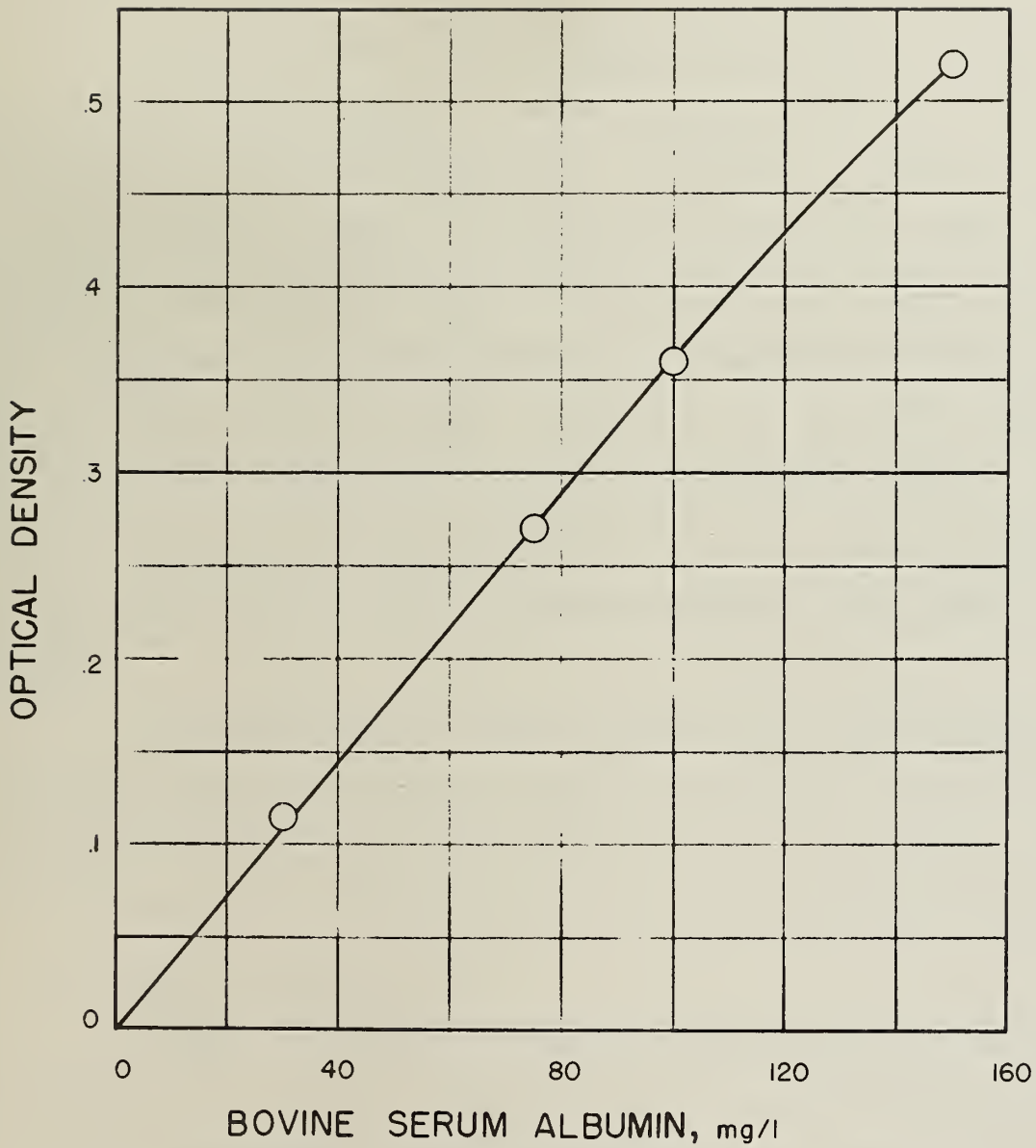


FIGURE 6. STANDARD CURVE FOR PROTEIN DETERMINATION, FOLIN'S METHOD

cell weight and the weight of TMG accumulated in 5 minutes, using cell concentrations up to 200 mg/ℓ and an initial substrate concentration of 5×10^{-4} M.

Deere (44) reported that E. coli cells unadapted to lactose showed a significant increase in lactase activity upon lysis. A similar observation was made by Lederberg (72). He obtained a 19-fold increase in the hydrolysis of ONPG when E. coli cells were ruptured. From these findings it may be assumed that tha kinetic behavior of intact cells toward ONPG depends upon the rate of transport of the substrate to the enzyme β-galactosidase within the cells. Therefore, the rate of ONPG hydrolysis by intact cells measures the activity of the galactoside permease, if the lysed cells are shown to have a higher ONPG activity. This rationale was used to measure the activity of galactoside permease in the present investigation.

The ONPG activity of intact and lysed cells was measured as described earlier. The cells were lysed by shaking 5 ml of the cell suspension with 0.1 ml toluene for 15 min (76).

8. Sludge volume index, SVI

Sludge volume index was determined from the volume occupied by sludge after 100 ml of mixed liquor was allowed to settle for 0.5 hr in a 100-ml graduated cylinder. The following relationship was used for calculation of SVI:

$$\text{SVI} = \frac{\text{ml of sludge volume}}{\text{percent suspended solids concentration in the mixed liquor}}$$

C. Experimental Technique and Equipment

1. Source of sludge

a. Glucose acclimated sludge

The sludge was obtained from a laboratory scale activated sludge unit operated on a fill and draw basis. The reactor used for this purpose was similar to that shown in Figure 11.

Aeration was achieved by dispersing compressed air at a pressure of approximately 15 psi through a carborundum diffuser. The rate of air flow was maintained between 0.1 and 0.2 ft^3/min per 2 ℓ. This rate of aeration was found to be sufficient to maintain a minimum dissolved oxygen concentration greater than 6 mg/ℓ. It also kept the activated sludge in suspension and in circulation. The system was maintained in a constant temperature water bath at 20° C.

The initial seed organisms were obtained from municipal sewage. Glucose, inorganic salts, and 200 ml of tap water were added to the unit, and the volume was made up to 2 liters with distilled water. The final concentration of the added compounds, exclusive of tap water, is shown in Table 1. These concentrations were adopted from the composition of a minimal medium suggested by Davis and Mingioli (77). The pH of the system was maintained at 7.0 by the addition of the phosphate salts. The procedure of wasting sludge and feeding is outlined below. After 11 hrs of aeration, the volume of the mixed liquor in the unit was measured and made up to 2 ℓ with distilled water, if any loss due to evaporation had occurred

TABLE 1

CONCENTRATION OF SUBSTRATE AND INORGANIC SALTS IN LABORATORY ACTIVATED SLUDGE UNITS

Compound	Concentration mg/ℓ
$C_6H_{12}O_6$ (Glucose)	1500
K_2HPO_4	1600
KH_2PO_4	790
$Mg\ SO_4 \cdot 7H_2O$	75
$(NH_4)_2 \cdot SO_4$	750
$FeSO_4 \cdot 7H_2O$	2.5
$ZnSO_4 \cdot 7H_2O$	2.5
$MnSO_4 \cdot 3H_2O$	2.5
$CaCl_2$	10

during the period of aeration, and 0.66 ℓ of the mixed liquor was then wasted. The remaining 1.34 ℓ of the mixed liquor was allowed to settle for 1 hr and then 0.66 ℓ of the supernatant was drawn off and discarded. The total volume of the mixed liquor in the unit was again made up to 2 ℓ with distilled water after adding the substrate, inorganic salts, and 200 ml of tap water. Aeration of the unit was then resumed.

During the course of the study, a bulking sludge condition developed on several different occasions. Microscopic examination of such a sludge always revealed a predominant growth of filamentous organisms. It is believed that these filamentous organisms are always present in mixed cultures developed on pure hexose sugars (18)(78). At times they dominate the flora of the system and cause bulking. The factors which give these organisms the advantage of successfully competing with others for food were not investigated. Under such conditions the unit was discarded and a new unit initiated from fresh sewage seed.

Usually after about a week of operation the concentration of biological solids in the unit attained equilibrium, i.e., the weight of net solids synthesized every 12 hrs was equal to the amount wasted. Figure 7 shows a typical change in the concentration of biological solids, COD, and glucose remaining in solution over a period of 11 hours, after the unit had attained equilibrium.

b. Lactose acclimated sludge

Lactose acclimated sludges were developed in two different manners. A sludge consisting predominantly of young cells was developed by aerating 20 ml of mixed liquor supernatant, obtained from the glucose unit described earlier, with 2 ℓ of medium containing 1000 mg/ℓ of lactose.

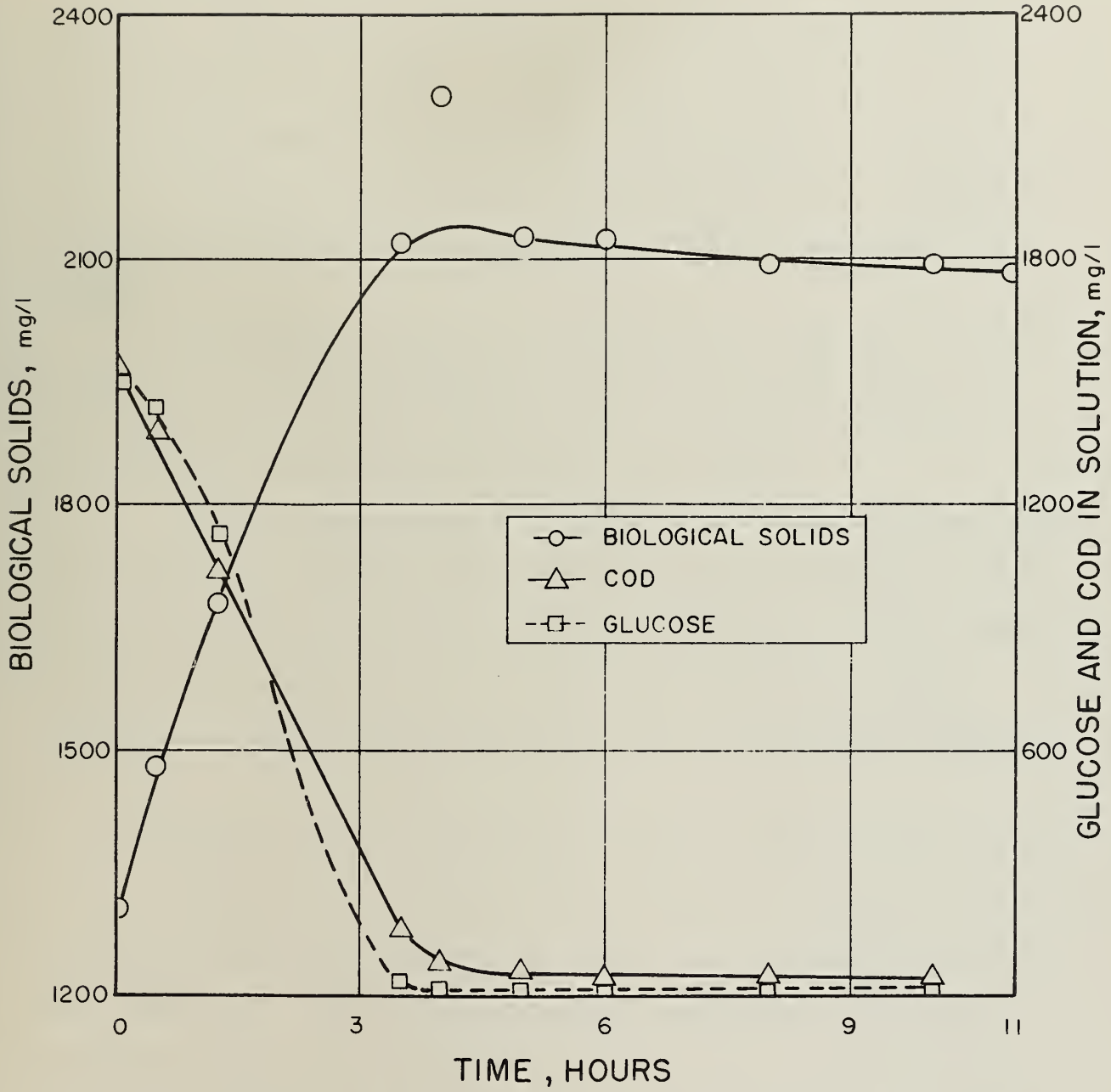


FIGURE 7. CHANGE IN BIOLOGICAL SOLIDS CONCENTRATION AND COD AND GLUCOSE IN SOLUTION OVER 11 HOURS, GLUCOSE SYSTEM

The concentrations of mineral salts in the medium were the same as shown in Table 1. The cells were harvested from the system when they entered the stationary phase of growth. At this time a complete removal of the substrate had occurred from the system. Figure 8 shows a typical growth curve, as followed by optical density measurements made at a wavelength of 540 mμ, and changes in the concentration of lactose remaining in the system.

The second type of sludge was developed in a fill and draw unit similar to that described for glucose. The initial seed organisms were obtained from the lactose acclimated young cell sludge.

In this system glucose was replaced by lactose in a concentration of 1000 mg/ℓ. The sludge obtained from this system was designated according to the number of days the unit had been in operation. Figure 9 shows the change in concentration of biological solids and COD and lactose remaining in solution over a period of 11 hrs on the 16th day of operation.

2. Studies of initial substrate uptake using glucose acclimated sludge

Sludge for these studies was obtained from the laboratory units described in the previous section. On the day of the experiment 660 ml of waste mixed liquor from the unit, containing approximately 2000 mg/ℓ of biological solids, was collected and allowed to settle for 0.5 hr at 3 to 5° C. Four hundred ml of supernatant was then drawn off and discarded. The settled sludge was then made up to a predetermined volume with distilled water containing mineral salts in concentrations as shown in Table 1, so as to give the required mixed liquor solids concentration.

For experiments in which the sludge concentration was to be held constant, while the substrate concentration was varied, a total volume of

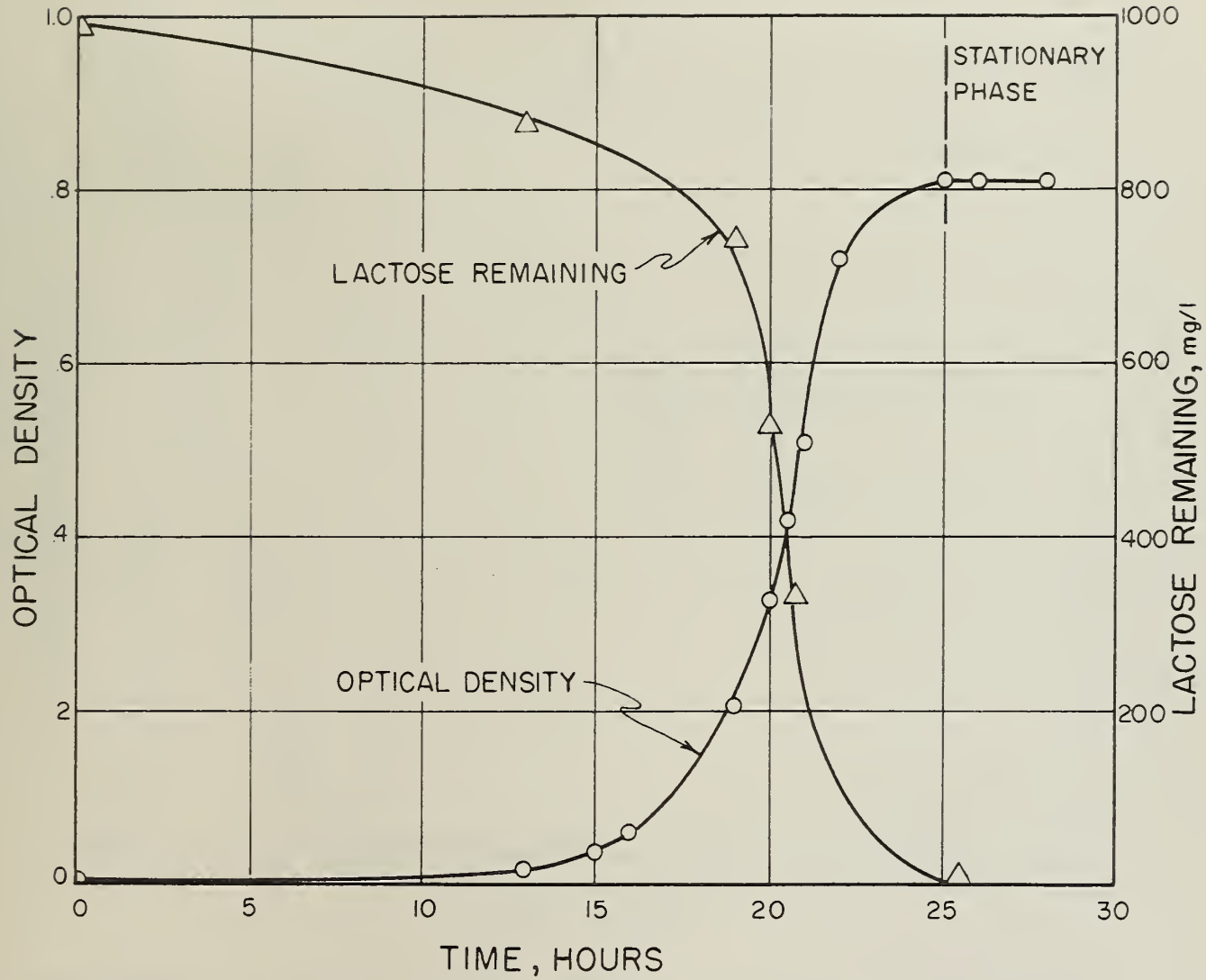


FIGURE 8. GROWTH OF LACTOSE ACCLIMATED ORGANISMS, SEEDED FROM GLUCOSE SLUDGE

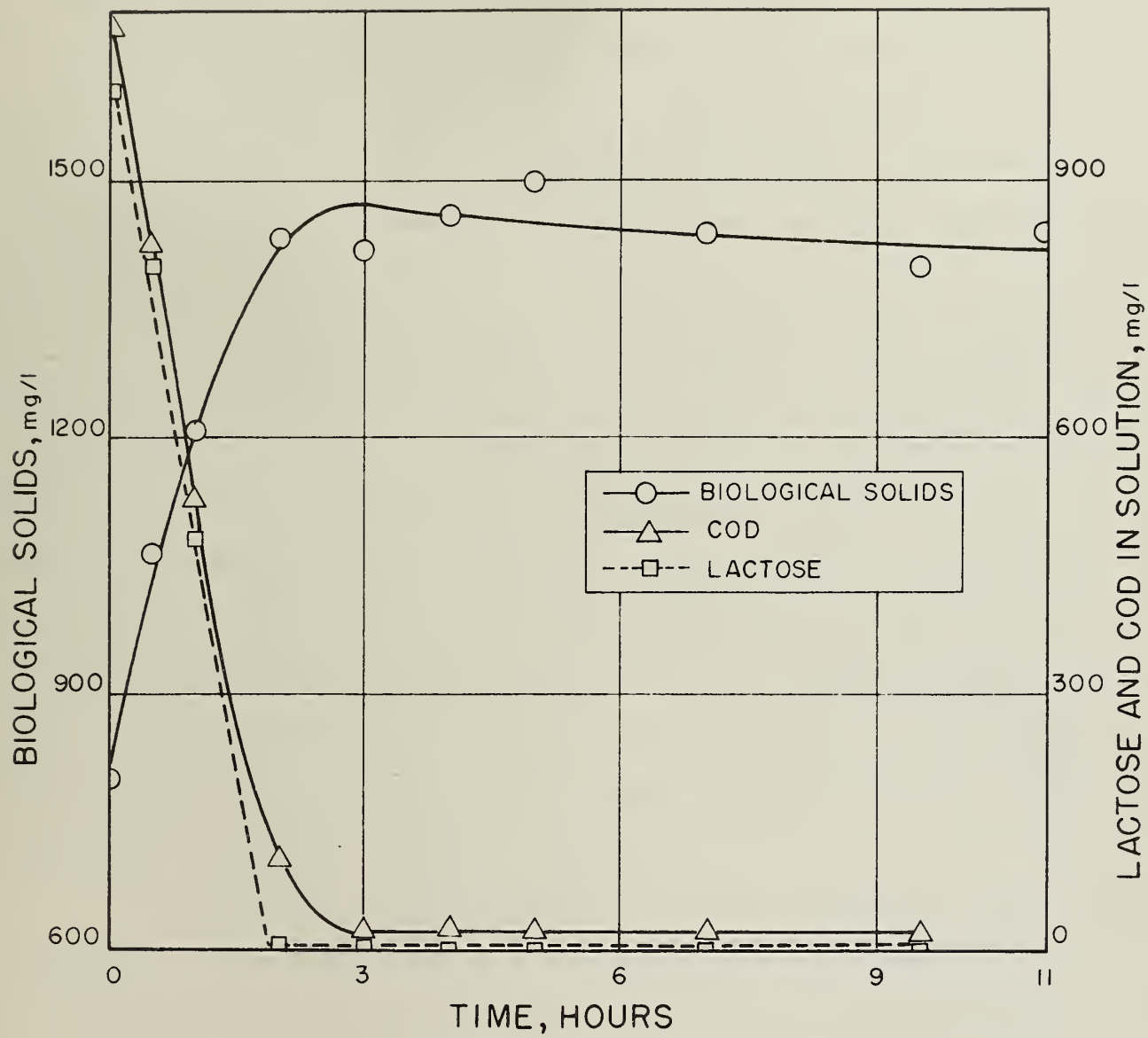


FIGURE 9. CHANGE IN BIOLOGICAL SOLIDS CONCENTRATION AND COD AND LACTOSE IN SOLUTION OVER 11 HRS., 16TH. DAY OF OPERATION, LACTOSE SYSTEM

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200 ml was adopted. The mixture was prepared with 100 ml of mixed liquor, distilled water with the mineral salts in it, and substrate stock solution. The volume of the water was adjusted according to the volume of the substrate stock solution. The substrate stock solution was prepared on the day of the experiment and had a concentration of 1000 mg/ℓ. The reaction vessels, 600-ml tall form beakers, with mixed liquor and water, were stored at 3 to 5° C.

Before initiating an experiment the reaction vessel was placed in a 20° C water bath and aerated for 20 min, except in the temperature studies when it was placed in a water bath maintained at the desired temperature. Air was supplied through a sintered glass diffuser. The air flow was maintained at 0.05 ft^3/min/200 ml. After the vessel had equilibrated at 20° C, the substrate stock solution was added. Samples of 10-ml volume were withdrawn at different time intervals and filtered through a 0.45 μ membrane filter disc. Only 10 seconds were allowed for filtration. The filtrate was stored at 3 to 5° C. It was later analyzed for the concentration of substrate remaining in solution.

An experiment usually required 4 to 6 hrs. Therefore, it was necessary to determine the effect of storage at 3° C on the activity of the sludge organisms. Freshly harvested sludge was washed and distributed in three beakers. Two of these were stored at 3° C. Rate of removal of substrate by sludge organisms was determined initially and after 5 and 10 hrs of storage. The results of the experiment are shown in Figure 10. It appears that storage at 3° C did not have any effect on the activity of the organisms.

In experiments where the sludge concentration was changed the volume was reduced to 100 ml as fewer samples were withdrawn. Culture

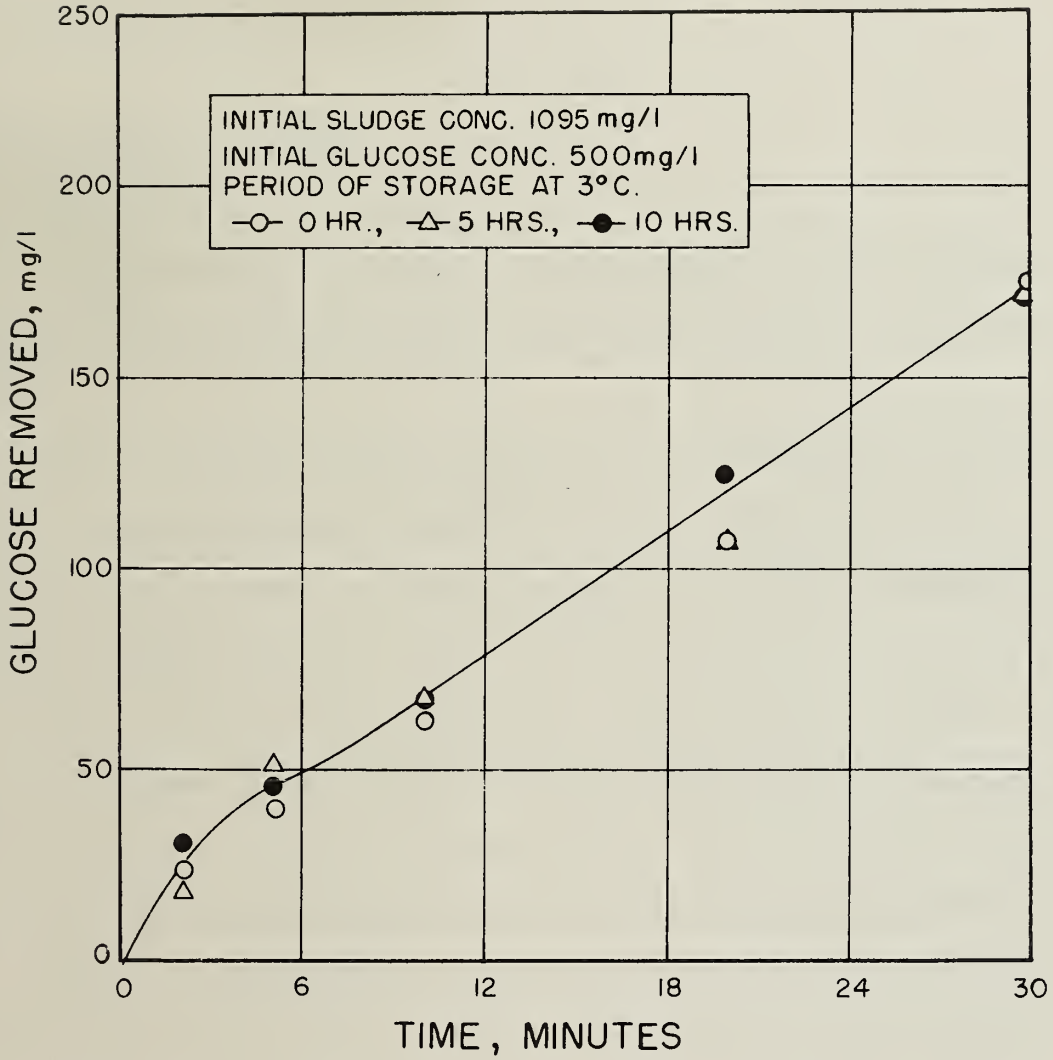

FIGURE 10. EFFECT OF STORAGE OF SLUDGE AT 3°C. ON GLUCOSE REMOVAL

tubes (38 x 200 mm) were used as reaction vessels. The procedure described above was also followed in these experiments except that the volume of sludge added to each reaction vessel was varied while the volume of the substrate solution remained constant.

3. Stabilization of sludge

a. Batch studies

These studies were carried out with lactose acclimated sludge. The sludge was obtained from the laboratory activated sludge unit described earlier.

Sludge was harvested from the units by centrifugation at 8000 xg for 10 min. It was resuspended in distilled water, containing mineral salts in concentrations shown in Table 1, using an Omin-mixer* homogenizer, at a Powerstat setting of 50 for 3 min. It was then made up to a predetermined volume to give a cell concentration of approximately 500 mg/ℓ. Lactose in a concentration of 1500 mg/ℓ was then added and the suspension was aerated for 0.5 hr. During this period 400 to 500 mg/ℓ of lactose was removed. After this initial period of contact the cells were separated from the medium by centrifugation, washed once with the mineral water, and resuspended as described above. All these operations were carried out at 3 to 5° C. This freshly fed sludge suspension was then aerated in the absence of an exogenous carbon source for 24 hours. During this period samples were withdrawn at different time intervals for analysis.

The sludge samples were analyzed for COD, protein, total carbohydrate, and trichloroacetic acid (TCA) soluble carbohydrate contents,

*Ivan Sorvall, Inc., Norwalk, Conn., U.S.A.

β-galactosidase activity and their capacity to remove glucose and lactose. The substrate removal capacity was determined by contacting 15 ml of sludge suspension with 15 ml of 200 mg/ℓ substrate solution for 15 min. The sludge was separated from the substrate by centrifugation, 10,000 xg for 10 minutes, in the case of young cells and by filtration through a 0.45 μ pore size membrane filter when sludge from the fill and draw unit was used. The substrate concentration was determined by the anthrone test. The TCA soluble fraction of the total carbohydrate content of the cells was extracted by mixing equal volumes of sludge suspension and 10 percent (w/v) TCA solution at 3 to 5° C. The other determinations were made directly after proper dilution.

b. Laboratory scale contact stabilization unit

A contact stabilization process was simulated by using a completely mixed tank fed on a batch basis. The period over whieh the substrate was removed was designated as the contact period, while the endogenous phase was designated as the stabilization period. The stabilization period was varied by changing the frequency of feeding.

Theory: In a completely mixed system there is no concentration difference from one part of the system to the other. Also if the reaction tank has an overflow the concentrations of substances coming out are the same as in the tank. A material balance for such a system receiving a uniform rate of flow can be set up as follows:

$$\text{Input} - \text{Output} = \text{Accumulation} \qquad 9$$

If the flow rate into the reactor is Q from a reservoir having a substrate

concentration of C_R, Equation 9 can be written as:

$$C_R \cdot Q - C_t \cdot Q = \frac{d(C_t \cdot V)}{dt} \qquad 10$$

where V is the tank volume and C_t is the concentration of the substrate in the tank at any time t.

If the flow to the tank ceases after a period T, and initially at t = 0, C_t = 0, Equation 10 on integration and substitution of these limits yields,

$$\frac{C_T}{C_R} = 1 - e^{\frac{-Q}{V} \cdot T} \qquad 11$$

where C_T is the concentration of substrate in the tank at t = T.

Similarly for the displacement of sludge from the tank by the inflowing feed, Equation 9 can be rewritten as

$$-S_t Q = \frac{d(S_t \cdot V)}{dt} \qquad 12$$

where S_t is the concentration of sludge in the tank at any time t.

If the sludge concentration in the reactor when the feeding is initiated (t = 0) is equal to S_o and S_T when the feeding ceases (t = T), Equation 12 on integration and substitution of these limits yields,

$$\frac{S_T}{S_o} = e^{\frac{-Q}{V} \cdot T} \qquad 13$$

Using Equations 11 and 13 the concentration of feed in the

reservoir and the rate and period of inflow of the feed can be calculated for a given set of operating parameters.

Design: The following operating parameters were adopted for the experimental system:

(i) $S_T = 1500$ mg/ℓ

(ii) $\frac{C_T}{S_T} = 0.4$ (Food to microorganism ratio)

(iii) $T = 1$ min

(iv) $V = 2$ ℓ

From (i) and (ii) above

$$\frac{C_T}{1500} = 0.4$$

or

$$C_T = 600 \text{ mg/ℓ}$$

Assuming the ratio of synthesis to respiration as one, we have

$$S_o = S_T + 1/2\ C_T$$

or

$$S_o = 1500 + \frac{600}{2} = 1800 \text{ mg/ℓ}$$

Substituting for S_o, S_t, t and V in Equation 13

$$\frac{1500}{1800} = e^{\frac{-Q}{2} \cdot 1}$$

or $$Q = 0.365 \text{ ℓ/min}$$

Again substituting for C_T, Q, T and V in Equation 11

$$600 = C_R \left(1 - e^{\frac{-0.365}{2} \cdot 1}\right)$$

or $$C_R = 3600 \text{ mg/ℓ}$$

As mentioned earlier the substrate consisted of a mixture of glucose and yeast extract in the ratio of 5:1 and mineral salts. The concentration of salts was the same as shown in Table 1 except for ammonium sulfate which was doubled because of a greater concentration of glucose in the feed solution. The COD of the medium was found to be the same as the sum of the concentrations of glucose and yeast extract.

Description: The experimental activated sludge unit is shown in Figure 11. The reactor consisted of a 4" internal diameter and 13" long Plexiglass cylinder, sealed at the bottom and having a removable cover plate at the top. It was provided with an adjustable overflow outlet. It had a 2 ℓ capacity with approximately 3.5" freeboard.

Air was supplied at a pressure of approximately 15 psi through a carborundum diffuser. To decrease the evaporation losses from the reactor, the air was first saturated by bubbling it through distilled water. The substrate was added to the reactor through a syphon from the feed reservoir. The rate of flow of feed through the syphon was adjusted by a screw clamp, while the total volume of feed was regulated by a solenoid valve. The valve was activated by a timer at preset intervals and

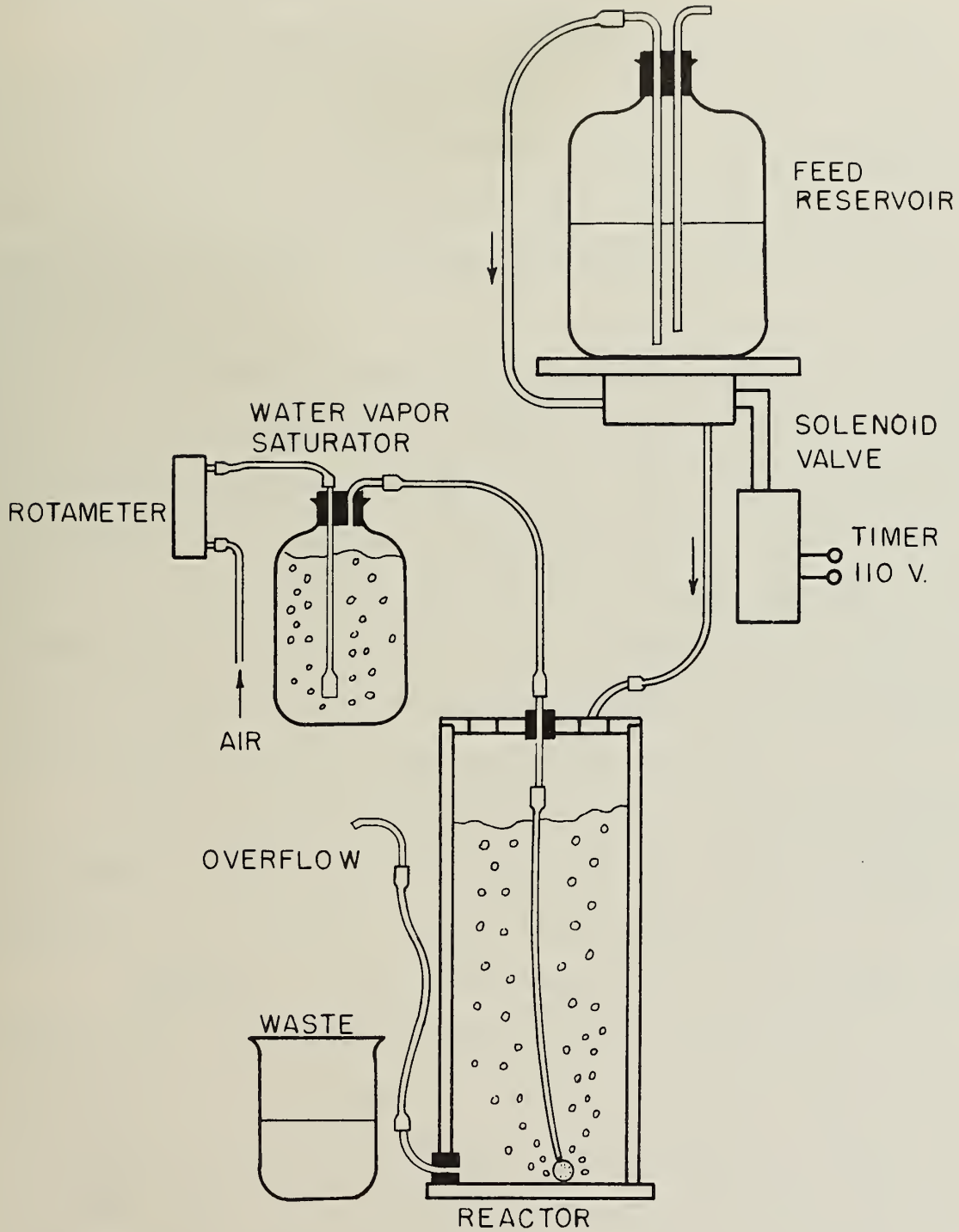

FIGURE 11. COMPLETELY MIXED ACTIVATED SLUDGE UNIT

was open for a period of 1 min at each feeding.

Hydraulic Performance: A mathematical analysis was made to calculate the volume and concentration of substrate in the feed to be added to the reactor to obtain a required loading. It was of interest to determine whether or not the laboratory unit could be considered as a completely mixed system. Two separate experiments were conducted to verify this.

To observe the rate of wasting of sludge, the reactor was filled with a solution of Eosin Y dye, of known strength. It was then fed distilled water at the rate of 365 ml/min, for 1 min. Composite samples of the effluent from the tank were collected over every 5-second periods. These were analyzed colorimetrically for the concentration of dye in them. To measure the progressive change in the concentration of substrate in the reactor, the experiment was repeated with distilled water in the reactor and dye solution of known strength in the feed reservoir.

The results of the experiment and expected theoretical curves for changes in sludge and substrate concentrations with time are shown in Figure 12. It is seen that they are in close agreement.

Operation: The reactor was seeded with 2 ℓ of activated sludge from the local sewage treatment plant. The unit was fed every 12 hours for twenty days at a loading rate of 0.4 mg of COD/mg of sludge solids. After this initial period of acclimation of the system, biological solids and sludge volume index of the overflow waste was determined every day. The concentration of biological solids in the reactor was computed according to the following equation:

$$\overline{S} = S_o \int_0^1 e^{\frac{-.365}{2} \cdot t} \, dt$$

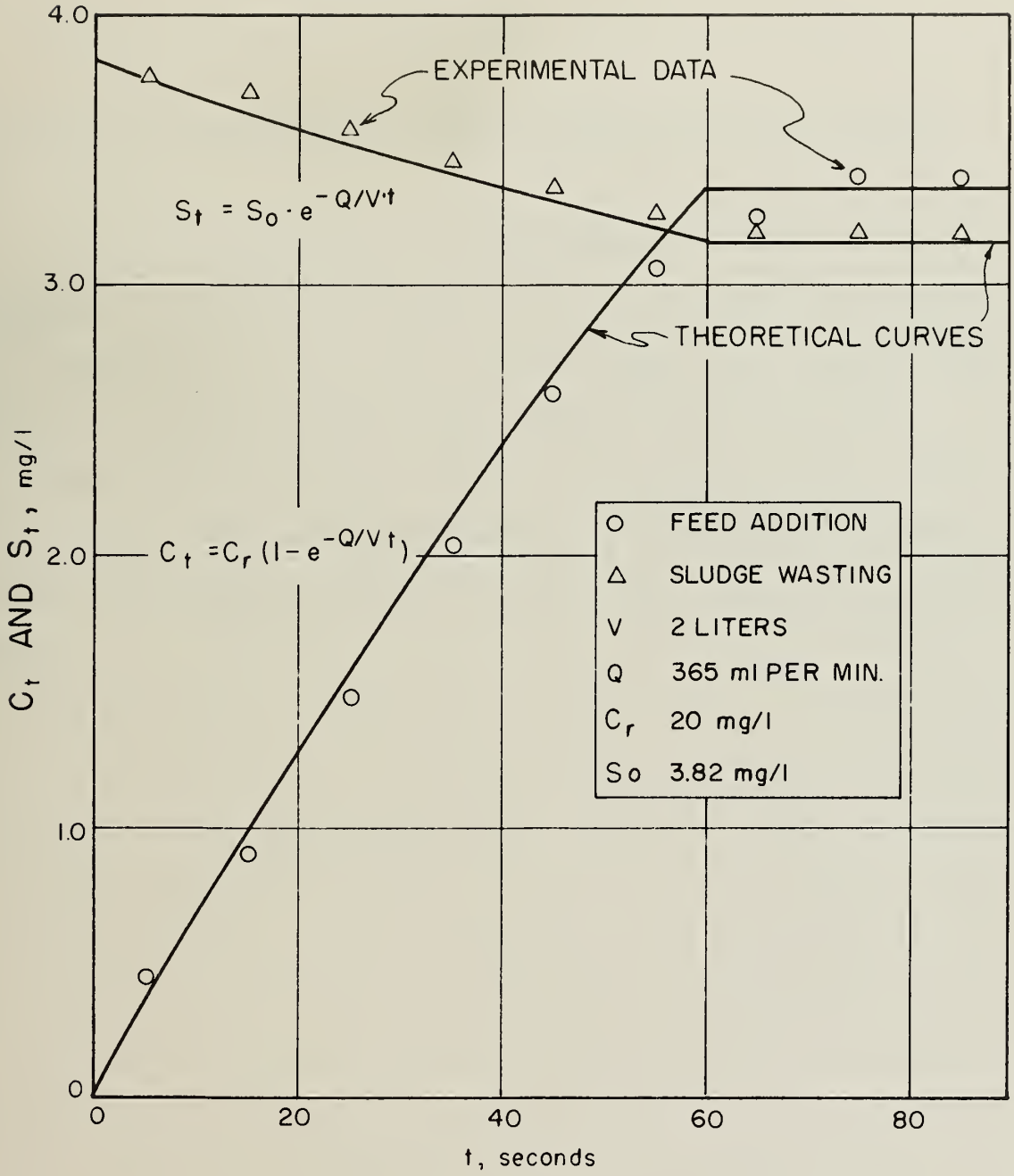

FIGURE 12. HYDRAULIC PERFORMANCE OF LABORATORY ACTIVATED SLUDGE UNIT

or $$S_o = 1.1 \overline{S}$$

where $\overline{S}$ is the concentration of biological solids in the total overflow waste.

Since it was desired to study the effect of loading rates on the performance of the system the frequency of feeding was gradually increased. Before increasing the loading rate to the next higher value, changes in the carbohydrate and protein content of the sludge and filtrate COD were measured over one feeding cycle. The system was allowed to come to equilibrium at a particular loading rate before the above determinations were made. For each loading rate at least 30 feed cycles were allowed for this purpose.

V. RESULTS AND DISCUSSION

Results of all experiments are presented in graphical form. In order to facilitate the presentation, a discussion of the results follows each phase of the experimental work. For the most part, only typical results are shown and discussed.

A. Studies of Initial Uptake of Substrate

1. Glucose substrate

Removal of glucose by acclimated sludge organisms at different food to microorganism (F/M) ratios is shown in Figure 13. The F/M ratio is defined as the ratio of the initial substrate concentration to the initial biological solids concentration. The different F/M values were obtained by feeding varying concentrations of glucose to sludge suspensions containing the same concentration of biological solids. After the addition of substrate, samples were taken and analyzed at one minute intervals for the first four minutes. Later on, the samples were more widely spaced. This was done in order to differentiate between the primary removal rate and the secondary removal rate which reflects the reactions of synthesis and respiration.

In Figure 13 it is seen that the primary removal approached equilibrium conditions within 3 to 4 minutes and then the rate of substrate uptake decreased. After 4 to 5 minutes the secondary rate became evident. Comparing the two removal rates at different F/M ratios, it is seen that both rates increased when the substrate concentration was increased from 200 to 500 mg/ℓ. With substrate concentrations above 500 mg/ℓ the

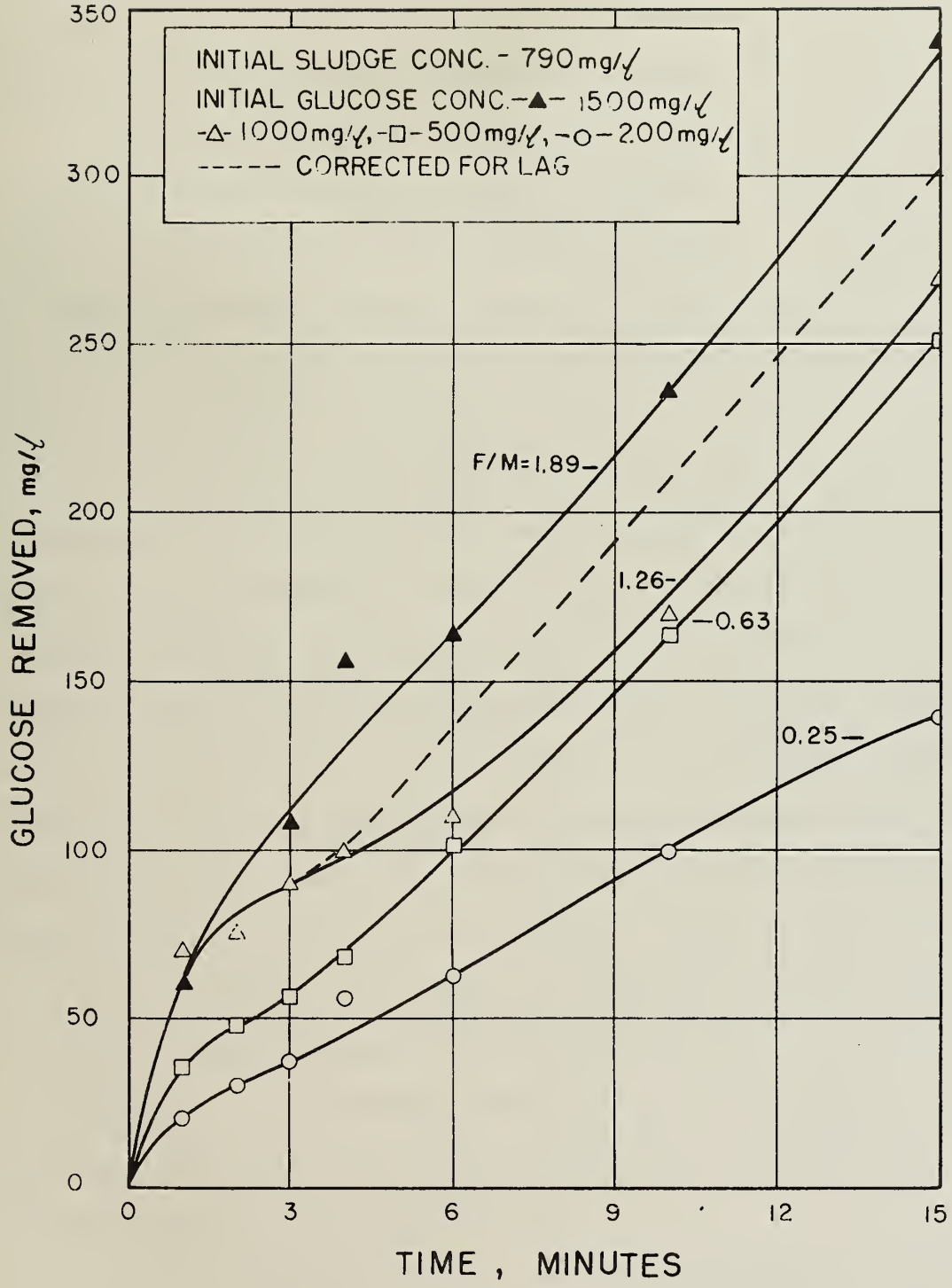

FIGURE 13. REMOVAL OF GLUCOSE BY ACCLIMATED SLUDGE AT DIFFERENT FOOD TO MICROORGANISM RATIOS

secondary rate did not increase, while the primary rate increased to a still higher value. However, increasing the substrate concentration above 1000 mg/ℓ did not result in any further change of the primary removal rate.

These observations can be explained if the removal mechanism is considered to follow the scheme outlined in Figure 1. According to this scheme the extracellular substrate is first transported to a substrate pool inside the cell by permease enzymes. The substrate concentration in this pool is in equilibrium with the outside substrate concentration according to Equation 6. The rate of transport of substrate to this pool is given by Equation 4, which increases with increasing substrate concentrations until the transport enzymes become saturated. An increase in substrate concentration above this saturating concentration does not result in an increase in the primary removal rate. However, the equilibrium level of the substrate concentration in the inside pool increases and the uptake continues at the same rate for a longer period of time to attain this level. Subsequent utilization of the substrate from the inside pool by hydrolyzing, respiratory and synthesizing enzymes, permits a further permeation of substrate into the cell. The magnitude of this secondary rate depends upon the rate limiting step in the metabolic scheme. The secondary rate increases with increasing concentration of substrate in the inside pool until the enzyme catalyzing the rate limiting step becomes saturated. Above this level, the concentration of the substrate in the pool has no effect on the secondary rate.

In the present experiment a substrate concentration between 500 and 1000 mg/ℓ resulted in the saturation of the primary removal rate and a concentration between 200 and 500 mg/ℓ resulted in the saturation of the secondary removal rate. It appears from the pattern of the curves that

the secondary removal started 3 to 4 minutes after the substrate had been added. For some unexplainable reason the lag was more pronounced for the system fed 1000 mg/ℓ of glucose. A removal curve, by neglecting the extended lag, has been included in the figure.

It should be noted that the data shown in Figure 13 can also be explained on the basis of an initial adsorption phenomenon, except for the saturation of the primary rate. The data have been plotted also according to the expression given by Weston and Eckenfelder (Equation 1, $L_{ri} = b\, L_o\, S_o$) in Figure 14. The plot does not conform to a straight line relationship as predicted by Equation 1. For higher values of $L_o \cdot S_o$, or L_o in this experiment, the removal was less. For 1 minute contact period, there was complete saturation of the removal mechanism at the values of $L_o \cdot S_o$ higher than 800×10^3. The substrate removed during longer contact periods is the sum of the concentration of the substrate accumulated inside and that utilized for synthesis and respiration. Since the concentration of substrate in the inside substrate pool depends upon the outside substrate concentration, a complete saturation of the overall removal rate is not possible.

The effect of different F/M ratios on the removal of substrate was also studied by changing the sludge concentration while the substrate concentration remained constant. The results of the experiment are shown in Figure 15. According to Equation 2 ($\frac{L_{ri}}{L_o} = 1 - e^{-K_i S_o}$) a plot of the log of the substrate remaining versus sludge concentration should produce a straight line. If the substrate is removed by an enzyme catalyzed reaction, however, the plot would tend to curve when sludge concentrations are low and the transport enzymes are saturated. The experimental data for 1-minute and 3-minute contact periods appear to conform to the latter

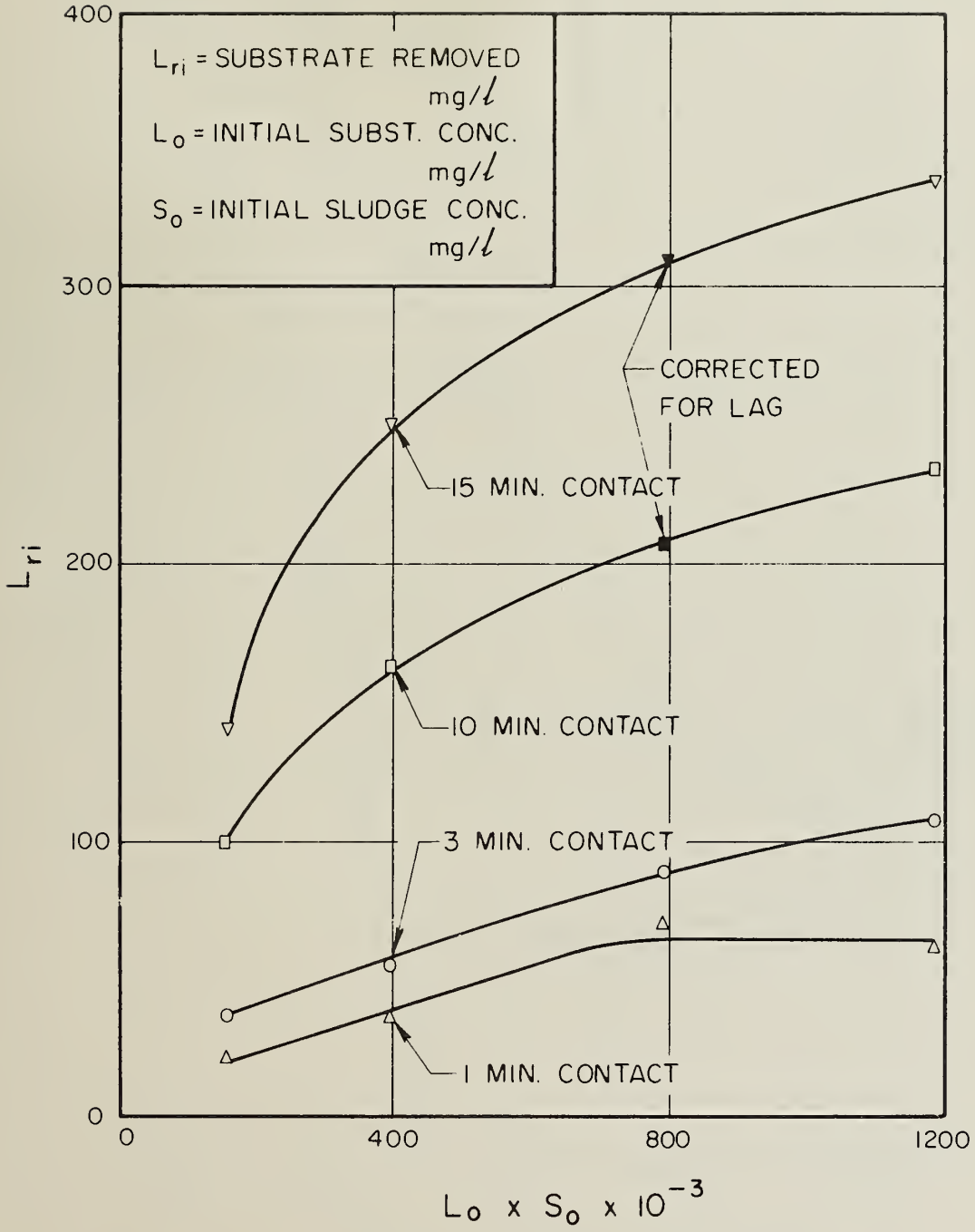

FIGURE 14. REMOVAL OF GLUCOSE BY ACCLIMATED SLUDGE IN DIFFERENT CONTACT PERIODS

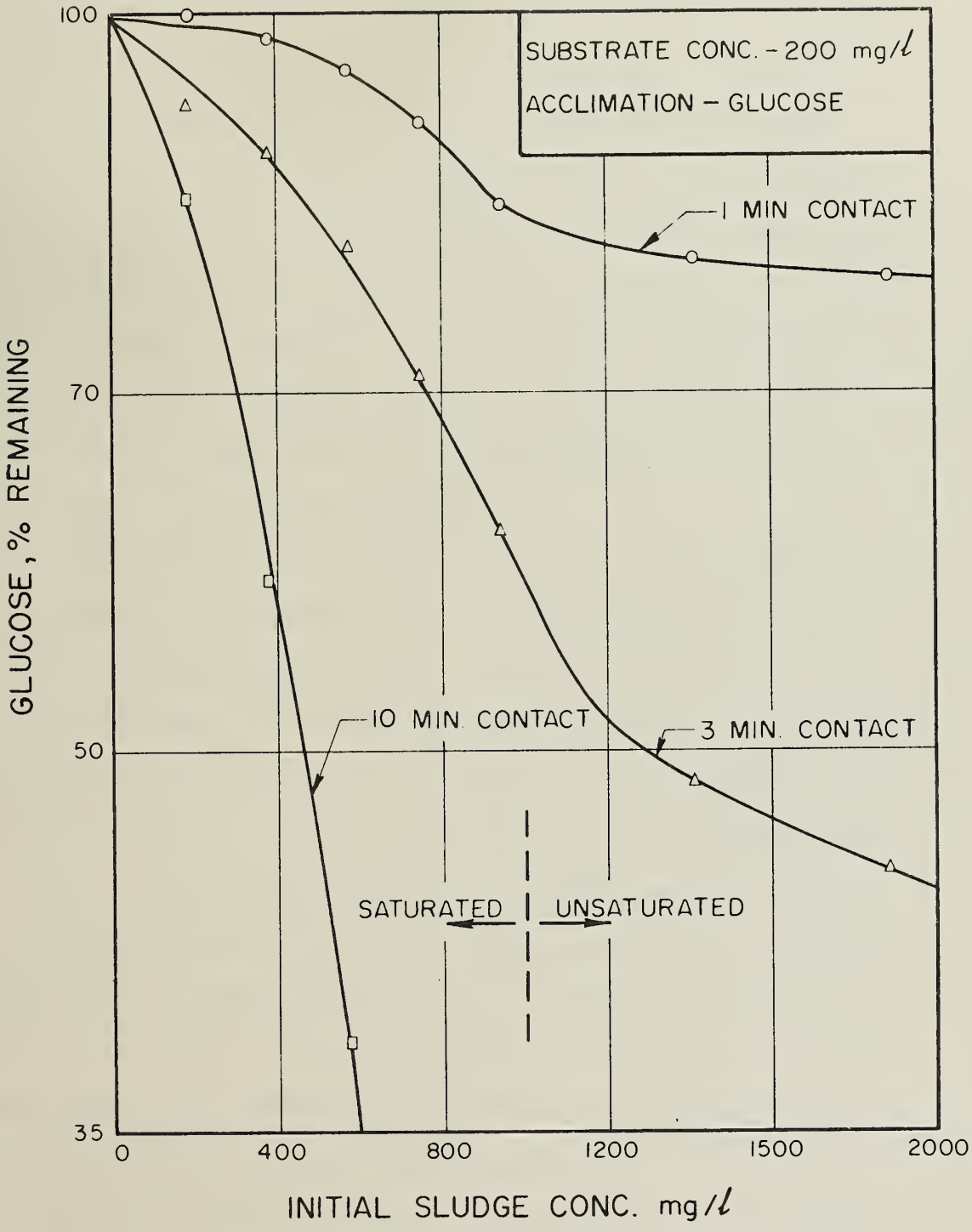

FIGURE 15. EFFECT OF SLUDGE CONCENTRATION AND CONTACT TIME ON GLUCOSE REMOVAL

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hypothesis. For sludge concentrations less than 1000 mg/ℓ, the plot deviates from a linear relationship showing the saturation of the enzymes. Below this concentration the amount of substrate removed is directly proportional to the sludge concentration and is independent of substrate concentration. Again for a 10-minute contact period a complete saturation of the overall uptake rate did not take place for the reason given earlier.

Comparing the results shown in Figure 13 and 15 it is seen that there was a difference between the F/M ratios at which the primary rate of removal became saturated. This is attributed to the inability of the laboratory activated sludge systems to produce populations identical in substrate removal activity from day to day.

In order to further investigate the nature of the substrate removal mechanism, substrate removal capacity of sludge under anaerobic conditions in a nitrogen atmosphere and of sludge boiled at 100° C, for 5 minutes, was determined. The results are shown in Figure 16. Observations on the removal of substrate by a control system are also included in the figure. It is seen that boiling of the sludge completely inhibited the removal. While anaerobic conditions did not interfere with the primary removal, it inhibited the secondary rate. These observations substantiate the argument that the primary removal is not a physical phenomenon but is carried out by biological means which can be denatured by high temperature. Furthermore, the energy required for the transport of substrate can be supplied by the organisms without requiring oxygen.

The initial removal mechanism was investigated also by studying the effect of temperature on the primary and secondary removal rates. The results of the experiment are shown in Figure 17. Increasing temperatures up to 30° C had a stimulatory effect on both primary and secondary removal

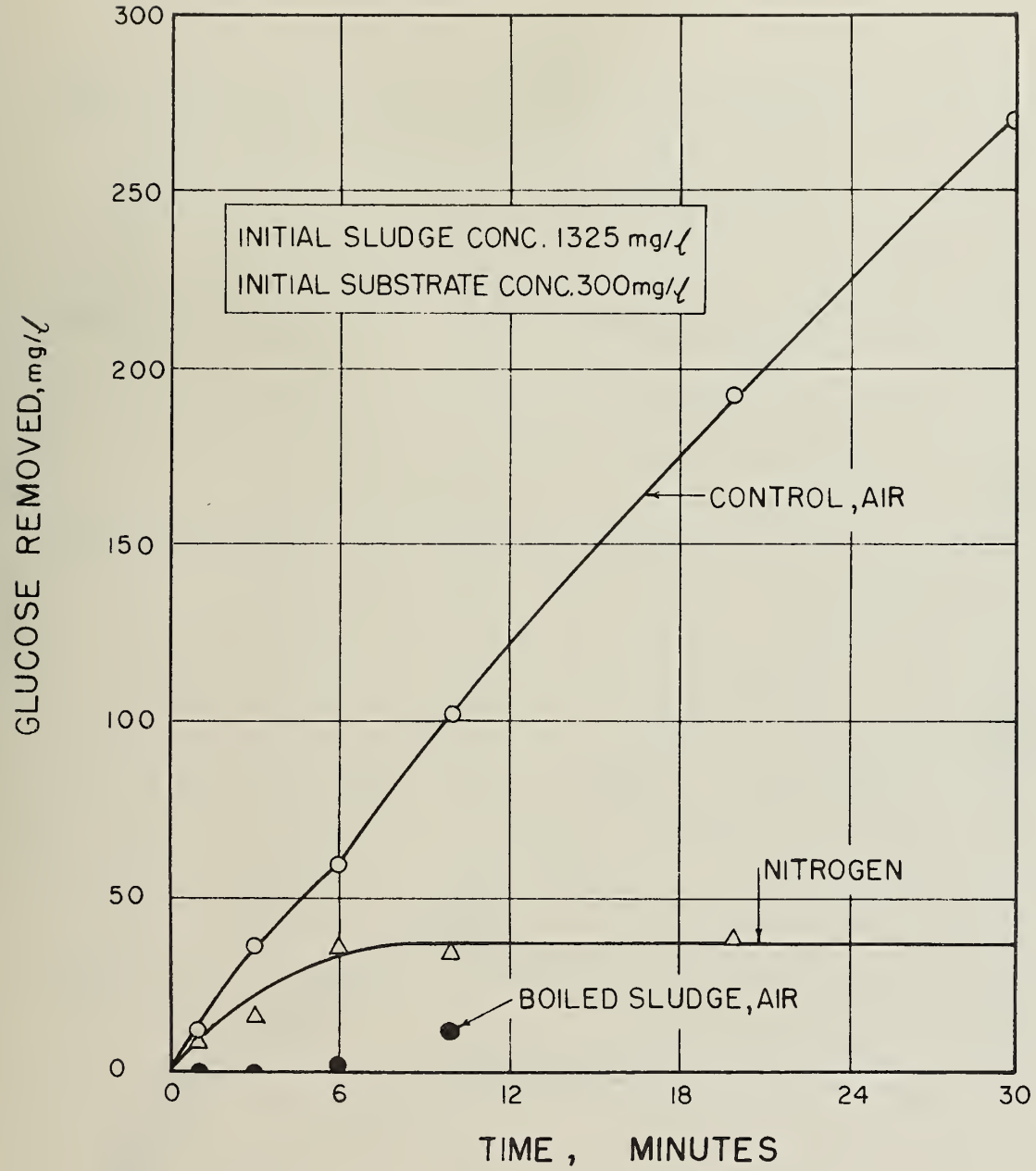

FIGURE 16. INITIAL REMOVAL OF GLUCOSE UNDER AEROBIC AND ANAEROBIC CONDITIONS BY ACCLIMATED SLUDGE AND BOILED SLUDGE

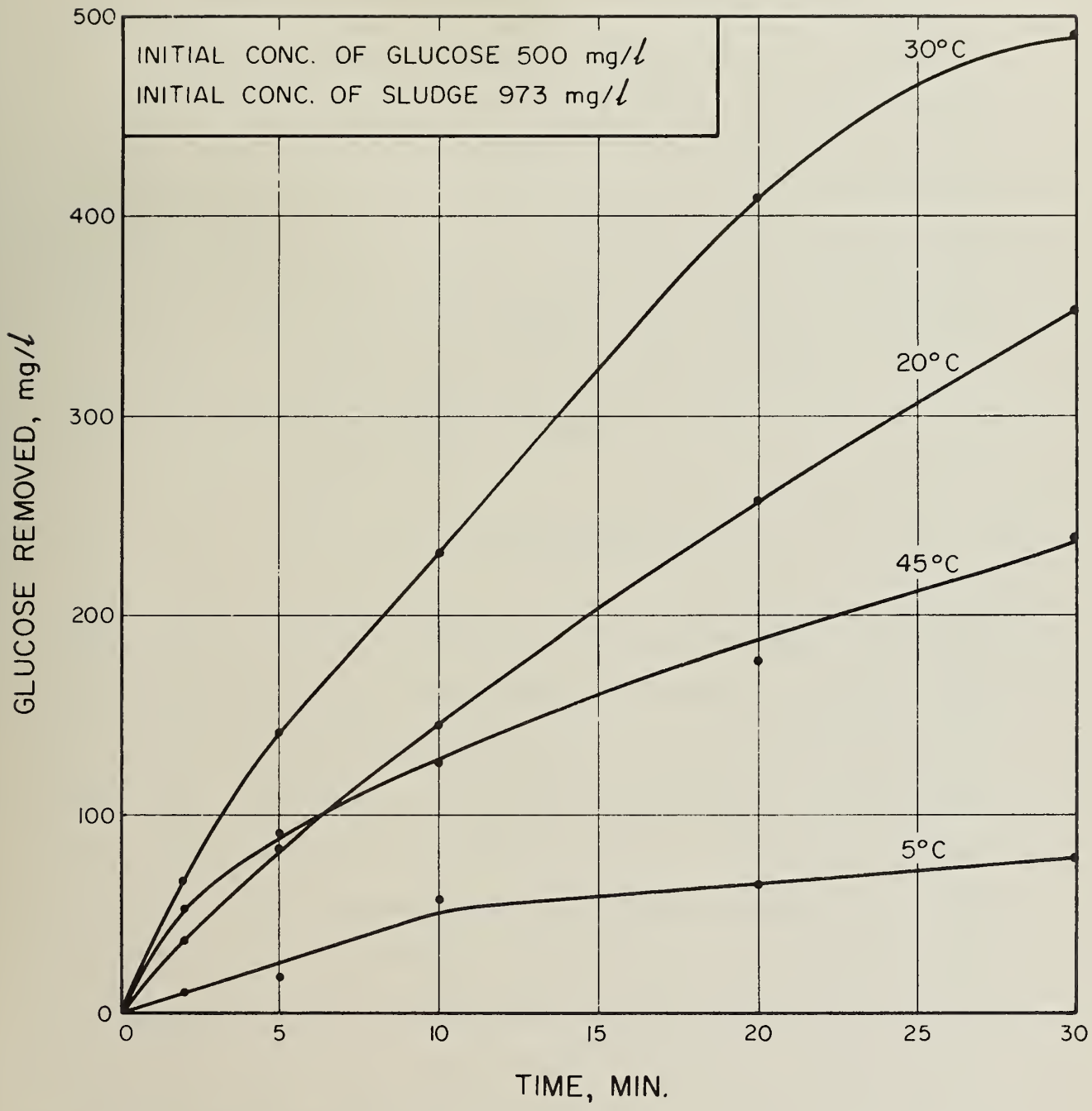

FIGURE 17. EFFECT OF TEMPERATURE ON REMOVAL OF GLUCOSE BY ACCLIMATED SLUDGE

rates. A further increase of temperature to 45° C resulted in a decrease of both the rates. The effect of different temperatures on the primary and secondary rates is shown in Figure 18. The primary rate was calculated on the basis of amount of glucose removed in the first 2 minutes. The secondary rate was calculated from the slope of the curves, in Figure 17, between the 14- and 20-minute intervals. From Figure 18 it is not possible to ascertain precisely the temperature of maximum activity due to lack of experimental data. However, a strong temperature dependence of both rates is evident. The rate of removal versus temperature relationship according to an Arrhenius plot (79) is shown in Figure 19. The Arrhenius equation can be written as follows:

$$\log \frac{K_2}{K_1} = \frac{\mu}{4.58} \left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad 14$$

where K_1 and K_2 are rates of removal at absolute temperatures T_1 and T_2, respectively, and μ is the Arrhenius constant. Therefore, a plot of log rate of removal versus reciprocal of absolute temperature should yield a straight line. From Figure 19 it is seen that above 40° C the rates fell off very rapidly and at lower temperatures also the curve deviated from a linear relationship. The shape of this plot which is linear between 10° and 35° C is typical of mesophilic biological reactions. Most biological reactions appear to have μ values between 8000 and 18000 cal for the linear portion of the curve (79). From the plots in Figure 19 μ values for the primary and secondary rates, between 20 and 30° C, were found to be equal to 10640 and 8550 cal, respectively. Using the 20° and 30° C data, the Q_{10} or the increase in the rate of uptake for each 10° C rise in temperature, was found to be 1.9 and 1.6 for the primary and secondary removal rates,

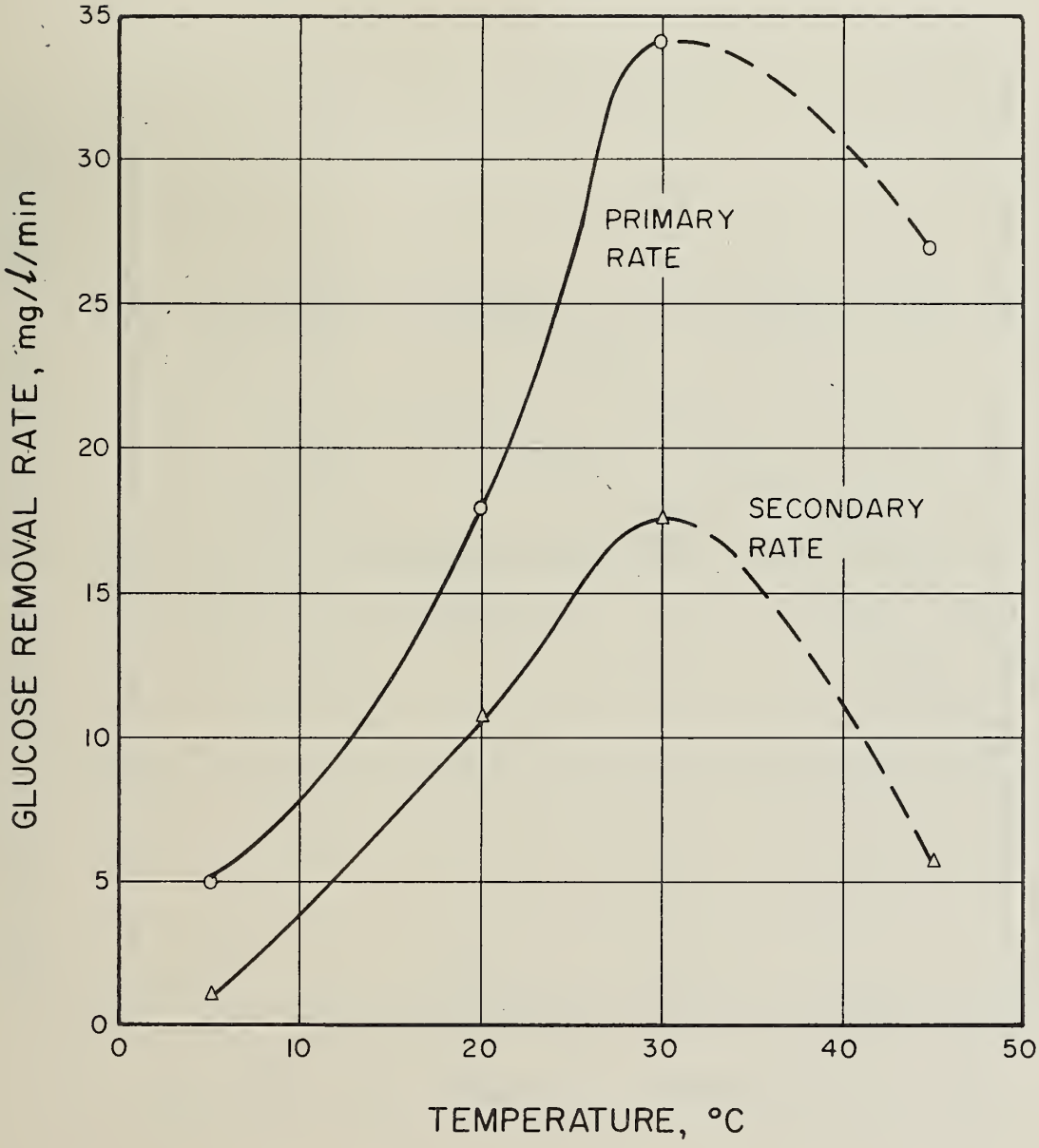

FIGURE 18. EFFECT OF TEMPERATURE ON RATES OF REMOVAL OF GLUCOSE BY ACCLIMATED SLUDGE

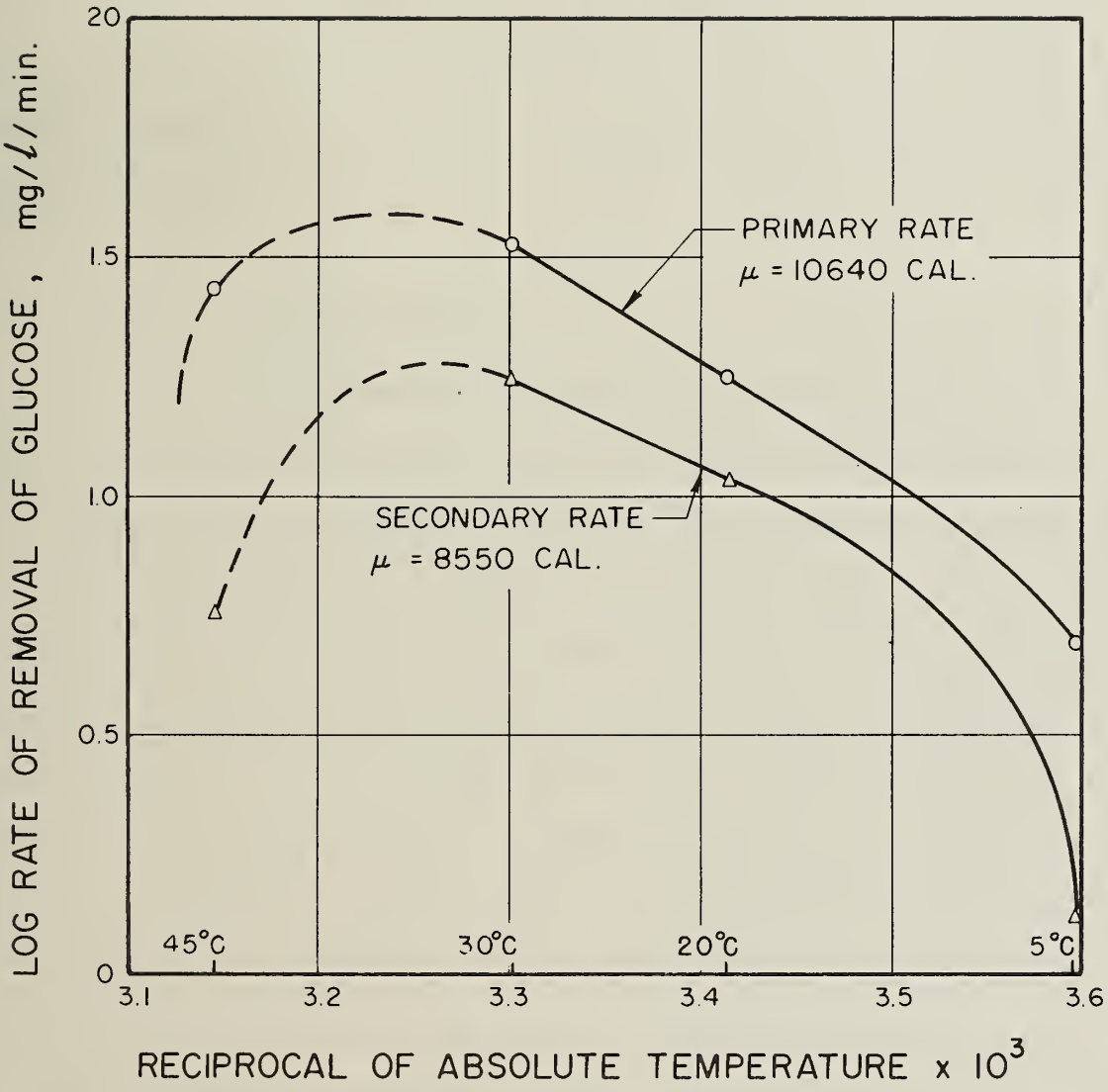

FIGURE 19. ARRHENIUS PLOT OF GLUCOSE REMOVAL RATES

respectively.

To summarize the above observations, it is seen that, like enzyme catalyzed reactions, both the primary and secondary rates can be saturated by increasing the F/M ratio. Furthermore, these rates are strongly dependent upon temperature. The relationship between change in rate and temperature follows the pattern of a typical biological reaction. These observations point towards the biological nature of the primary rate of substrate removal.

2. α-Methyl glucoside substrate

In the studies described above, using glucose substrate, the primary rate of substrate transport into the cell was influenced and partially masked by the secondary rate resulting from a simultaneous metabolism of the accumulated substrate. Therefore, a complete analysis of the primary rate, according to the Equation 4 which describes the rate of transport of substrate by permease enzyme systems, was not possible. References were cited in Chapter 2 in which α-methyl glucoside was used successfully as the substrate in pure culture studies to study the transport of substrate into the cells. It should be recalled that E. coli concentrated this substrate inside but did not metabolize it. Since in the present investigation, a heterogeneous population was under study, it was necessary initially to establish its response to α-methyl glucoside. Figure 20 shows the oxidation of equimolar solutions of glucose and α-methyl glucoside by glucose acclimated sludge. While oxygen was consumed at a higher rate for glucose substrate as compared to the endogenous respiration rate from the start, there was no difference for the first hour with α-methyl glucoside as the substrate. After this initial lag the rate of respiration increased and

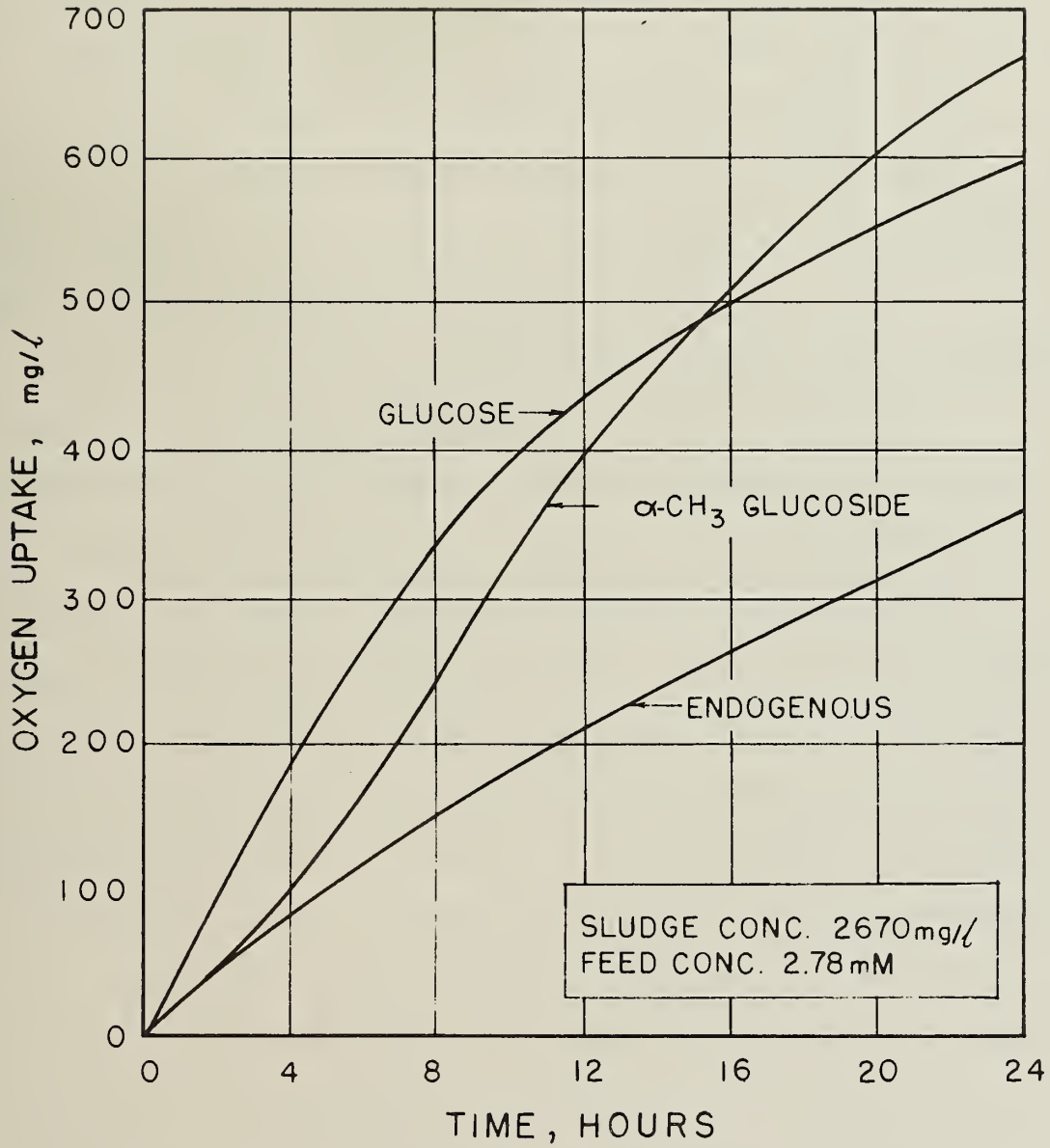

FIGURE 20. OXIDATION OF EQUIMOLAR SOLUTIONS OF GLUCOSE AND α-METHYL GLUCOSIDE BY GLUCOSE ACCLIMATED SLUDGE

was the same as that for the glucose system after 6 hrs. After about 24 hrs the oxygen uptake rate for both systems became parallel to the endogenous respiration rate. The total oxygen uptake for the α-methyl glucoside system was more than that for glucose system. This shows that the methyl moiety of α-methyl glucoside was probably also being utilized by the organisms. It was concluded from this experiment that the organisms, acclimated to glucose, initially did not have the necessary α-glucosidase enzymes and required a period of induction.

Since there is evidence that both glucose and α-methyl glucoside are transported by the same permease system, the above observations support the possibility that α-methyl glucoside was concentrated inside the cell during the time required for induction of the α-glucosidase. Figure 21 shows the removal of equimolar solutions of glucose and α-methyl glucoside by glucose acclimated sludge. It is seen that the sludge organisms rapidly removed and probably accumulated α-methyl glucoside in the first four minutes and an equilibrium was established. After about 15 minutes more glucoside was taken up at a different rate. It is believed that this secondary removal reflected the synthesis of α-glucosidase within the cells. The glucose removal followed the same pattern as shown in Figure 13 and discussed earlier. On the basis of the data shown in Figure 21 and other similar experiments, it was decided to take the amount of α-methyl glucoside removed within the first 15 minutes as the equilibrium level of the inside substrate pool. It was also concluded that the secondary rate, representing respiration and synthesis, did not interfere with the primary rate of removal during this period.

Removal of α-methyl glucoside by glucose acclimated sludge at different F/M ratios is shown in Figure 22. This experiment was analogous

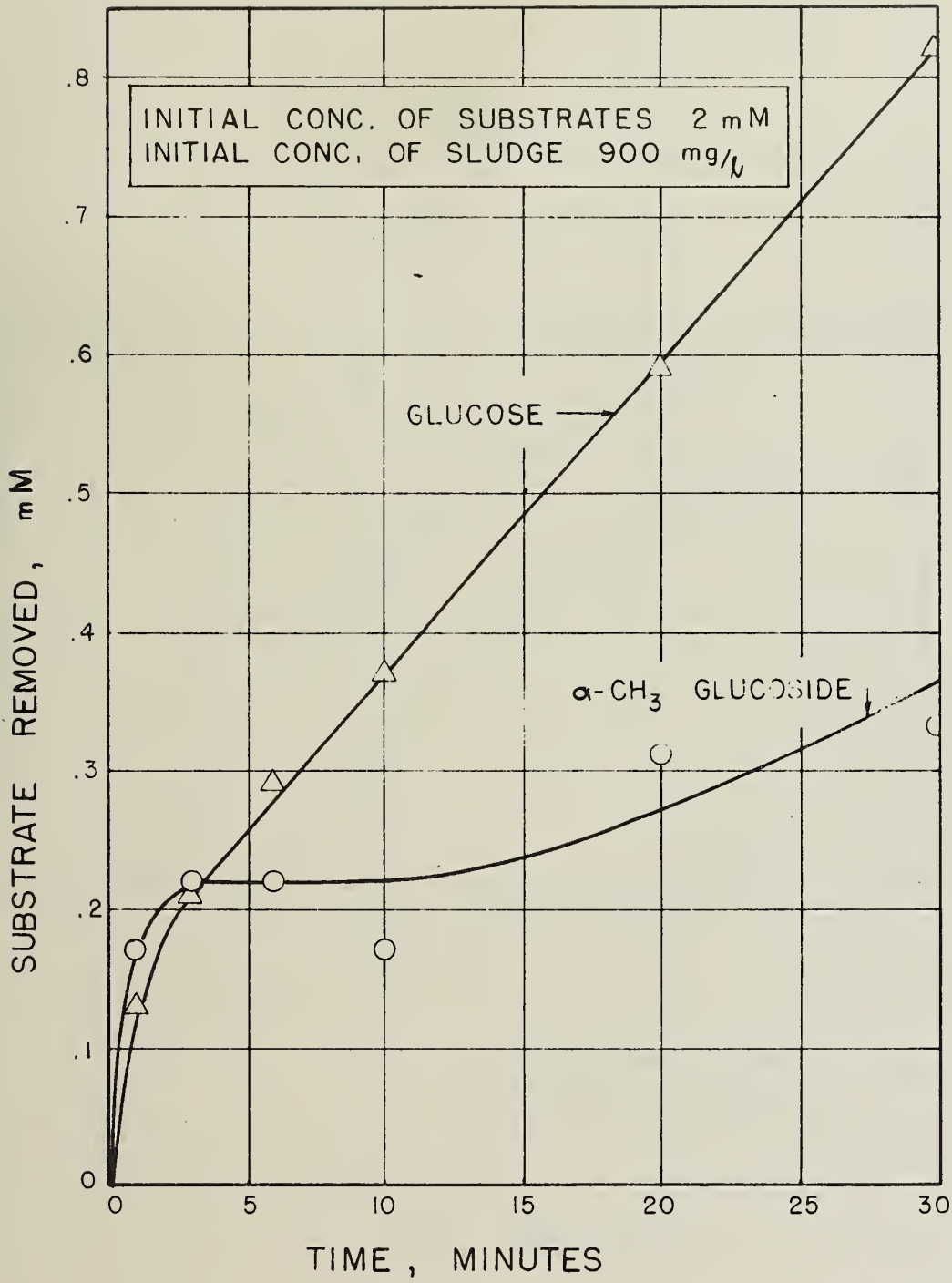

FIGURE 21. INITIAL REMOVAL OF EQUIMOLAR SOLUTIONS OF GLUCOSE AND α-METHYL GLUCOSIDE BY GLUCOSE ACCLIMATED SLUDGE SOLIDS

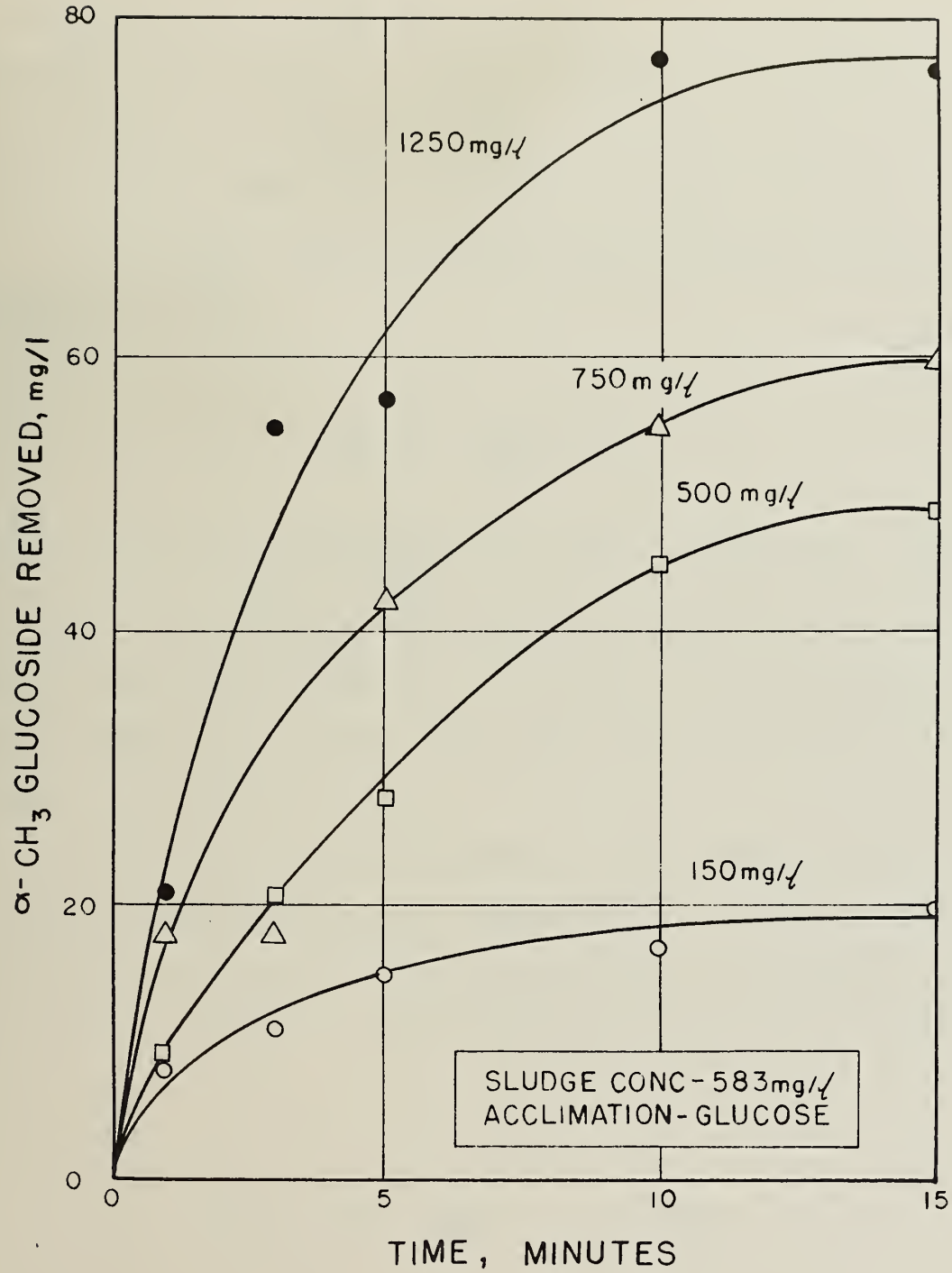

FIGURE 22. INITIAL REMOVAL OF α-METHYL GLUCOSIDE AT DIFFERENT FOOD TO MICROORGANISM RATIOS

to the glucose substrate experiment (Figure 13). It is seen that the experimental data do not show a complete saturation of the primary rate. The rate appeared to be higher for systems with greater substrate concentration. All the systems reached equilibrium in about 15 minutes.

The Langmuir adsorption isotherm, given by Equation 3, can also be written as follows:

$$\frac{1}{G_{ex}} = \frac{K_m}{Y} \cdot \frac{1}{G_{in}^{eq}} + \frac{1}{Y} \qquad 15$$

Therefore a plot of $\frac{1}{G_{ex}}$ versus $\frac{1}{G_{in}^{eq}}$ should yield a straight line. Figure 23 shows a plot of the 15-minute uptake values versus external substrate concentration according to Equation 15. The values of K_m and Y, which are given by intercepts of the x and y axes, were found to be equal to 833 and 130 mg/ℓ, respectively.

Both adsorption and the enzymatic transport mechanism predict identical equilibrium relationships (Equations 3 and 6). However, the velocity of reaction, which leads to the equilibrium, is different. The rate of uptake according to Langmuir's adsorption theory can be written as:

$$\frac{dG_{in}}{dt} = k_1 \left(1 - \frac{G_{in}}{Y}\right) G_{ex} - k_2 \cdot \frac{G_{in}}{Y} \qquad 16$$

where k_1 and k_2 are rate constants and $\frac{k_2}{k_1}$ as given by equilibrium condition is equal to K_m. Equation 16 upon integration and substitution yields:

$$\log \left(1 - \frac{G_{in}}{Y} - \frac{K_m G_{in}}{G_{ex} Y}\right) = - \frac{k_1}{Y} (G_{ex} + K_m) \cdot t \qquad 17$$

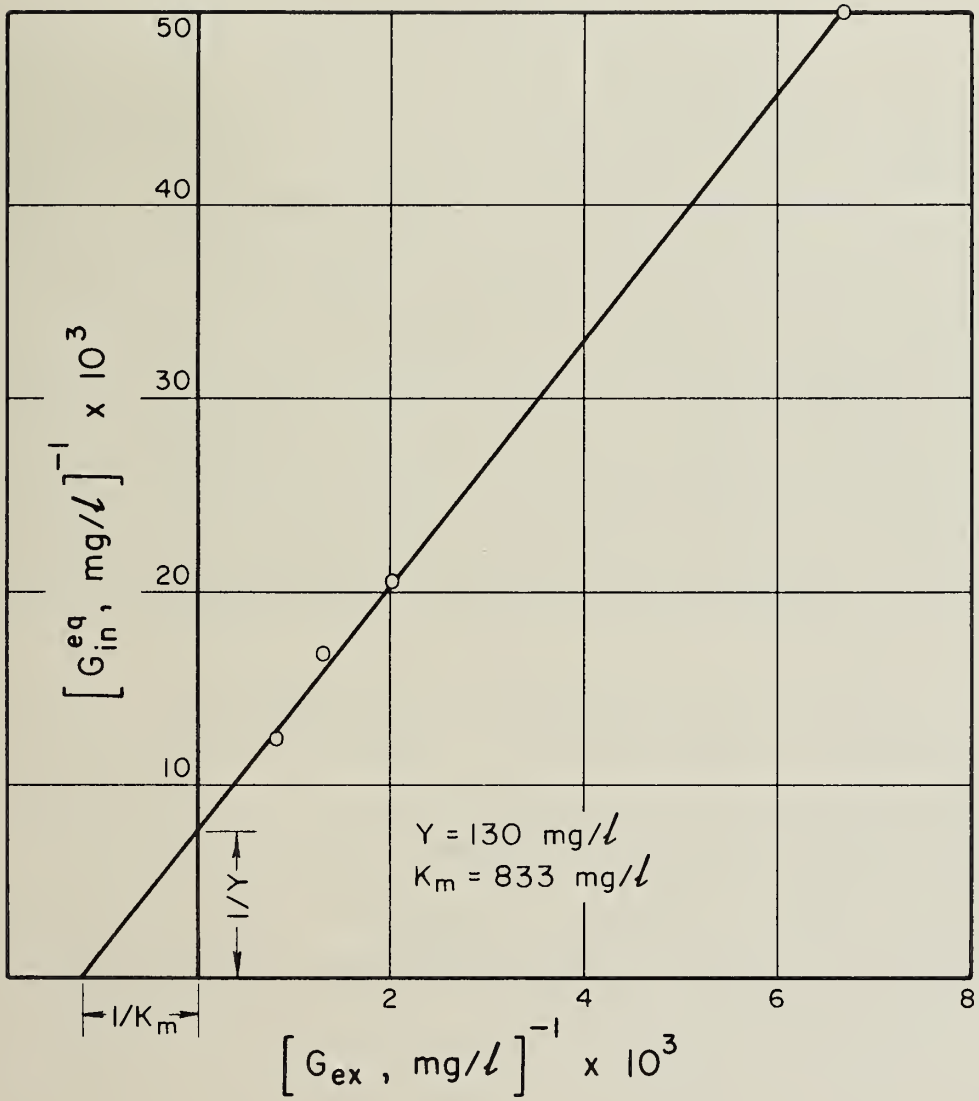

FIGURE 23. ACCUMULATION OF α-CH_3 GLUCOSIDE AS A FUNCTION OF EXTERNAL SUBSTRATE CONC., RECIPROCAL COORDINATES

The time course of substrate removal according to an enzymatic reaction, as mentioned earlier, is given by the following equation,

$$G_{in} = \frac{V_{in}^{max}}{k_{ex}} \cdot \frac{G_{ex}}{K_m + G_{ex}} (1 - e^{-k_{ex}t}) \qquad 5$$

In this equation $\frac{V_{in}^{max}}{k_{ex}} \cdot \frac{G_{ex}}{K_m + G_{ex}}$ can be replaced by G_{in}^{eq}.

The theoretical uptake curves, given by Equations 5 and 17, for the variables of the present experiment, and the experimental data are shown in Figure 24. The constants K_m and Y in the two equations were obtained from Figure 23. The constant k_1 in Equation 17 was calculated by substituting the G_{in} value at 1 minute for the 150 mg/ℓ substrate concentration system (Figure 22). The constant k_{ex} in Equation 5 was obtained from the slope of the straight line plot shown in Figure 25. The plot is obtained by rearranging Equation 5 in the following manner,

$$\log (G_{in}^{eq} - G_{in}) - \log G_{in}^{eq} = -k_{ex}t \qquad 18$$

The 150 mg/ℓ substrate concentration system was chosen to calculate these constants for two reasons: (i) a greater reliance could be placed on the experimental data of this system as compared to others because of the lower substrate concentration which required lower dilution for the purpose of analysis and (ii) to avoid a saturated initial velocity at higher substrate concentrations, which would have resulted in an erroneous k_1 value.

It is seen from Figure 24 that the adsorption theory, or a similar hypothesis which predicts a direct relationship between velocity of uptake and substrate concentration, yields higher initial velocities of uptake as

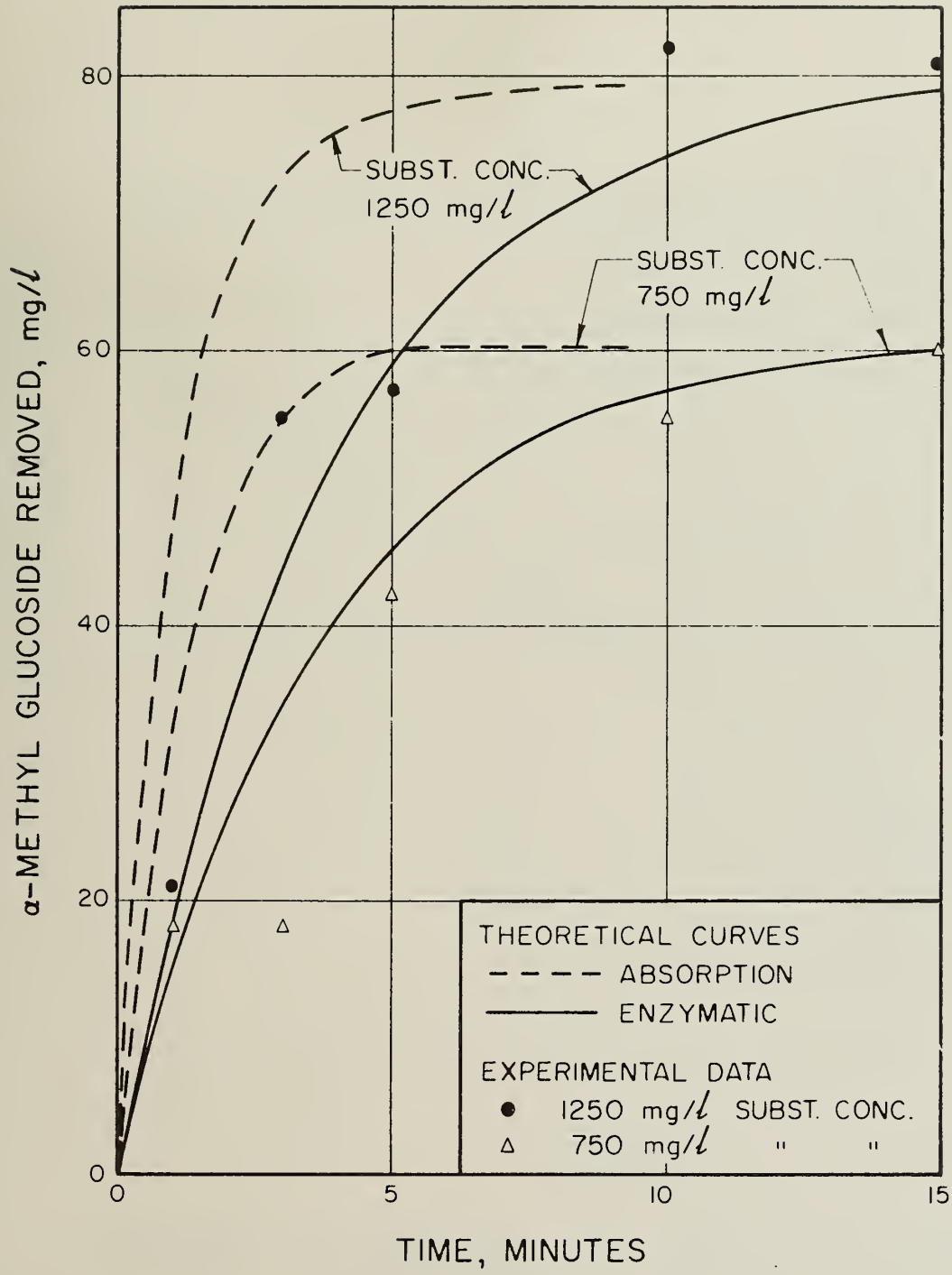

FIGURE 24. COMPARISON OF OBSERVED REMOVAL OF α-CH_3 GLUCOSIDE BY GLUCOSE ACCLIMATED SLUDGE WITH THEORETICAL CURVES

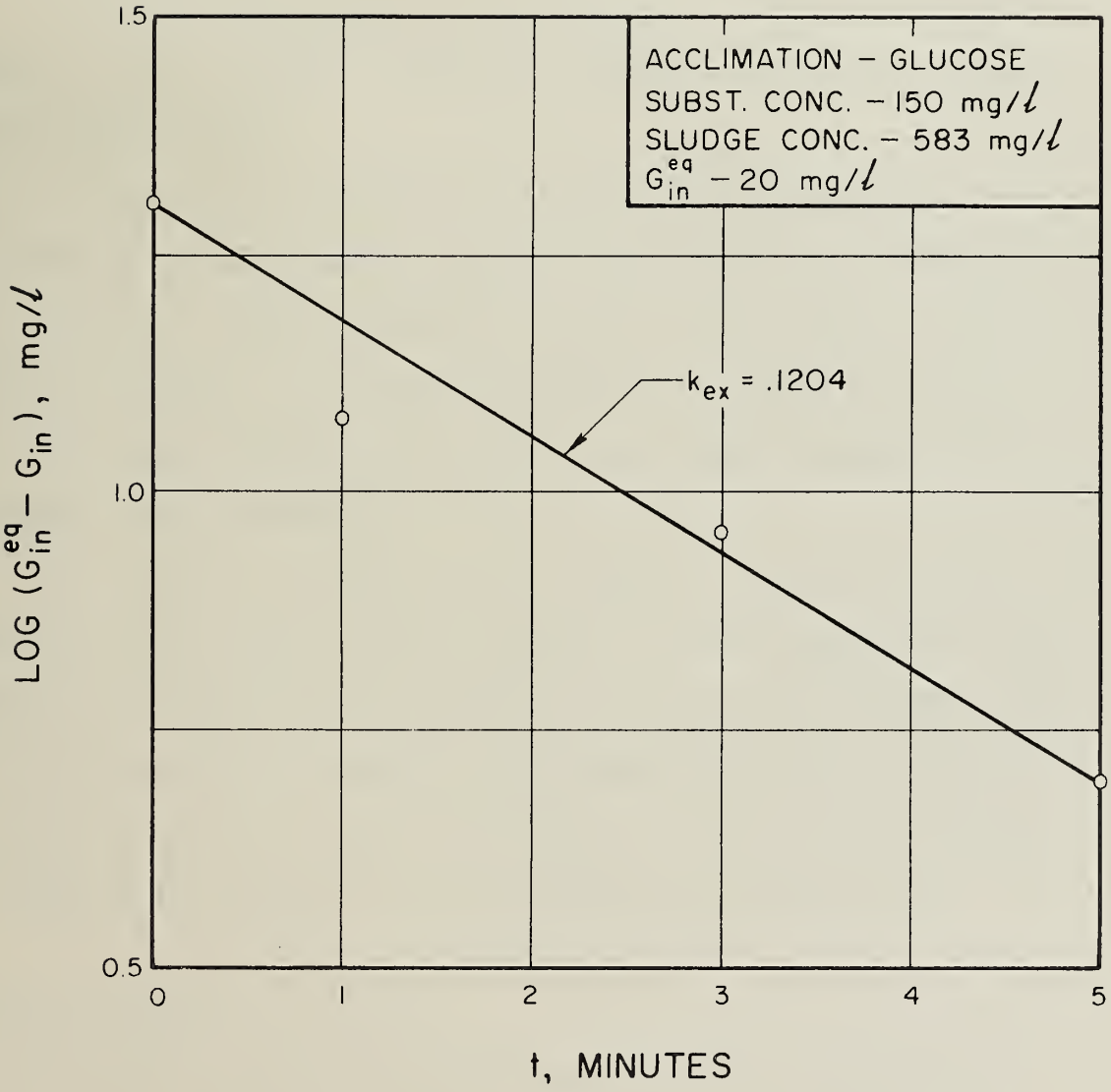

FIGURE 25. REMOVAL OF α-CH_3 GLUCOSIDE, ACCORDING TO EQUATION 18

compared to enzymatic transport. Also the experimental data appear to agree with the enzymatic transport model. In the analysis of data given here, the sludge concentration was not taken into account as it was the same in all systems.

The equilibrium data were also fitted to a Freundlich adsorption isotherm. The data deviated considerably from the relationship at higher substrate concentrations.

The results of an experiment in which sludge concentration was varied while the concentration of α-methyl glucoside was held constant are shown in Figure 26. These results are similar to those shown in Figure 15 for glucose substrate. It appears that 400 mg/ℓ of substrate was sufficient to saturate the primary uptake rate of the highest concentration of sludge used in this experiment.

In summary, the primary rate of removal of substrate could be separated from the secondary rate by the use of α-methyl glucoside as the substrate. The primary rate was found not to be a direct function of the substrate concentration as predicted by an adsorption model or existing formulations for contact removal of substrates. It followed the kinetics of an enzymatic reaction and could be saturated at high F/M ratios.

B. Sludge Stabilization Studies

Previous investigations describing an initial increase in BOD removal rate by sludge organisms followed by a decrease with increasing stabilization periods were cited earlier. The experiments reported in this section were designed to delineate this observation. All experiments were conducted with lactose acclimated sludge. Stabilization was initiated after an initial contact period of 0.5 hr with the substrate.

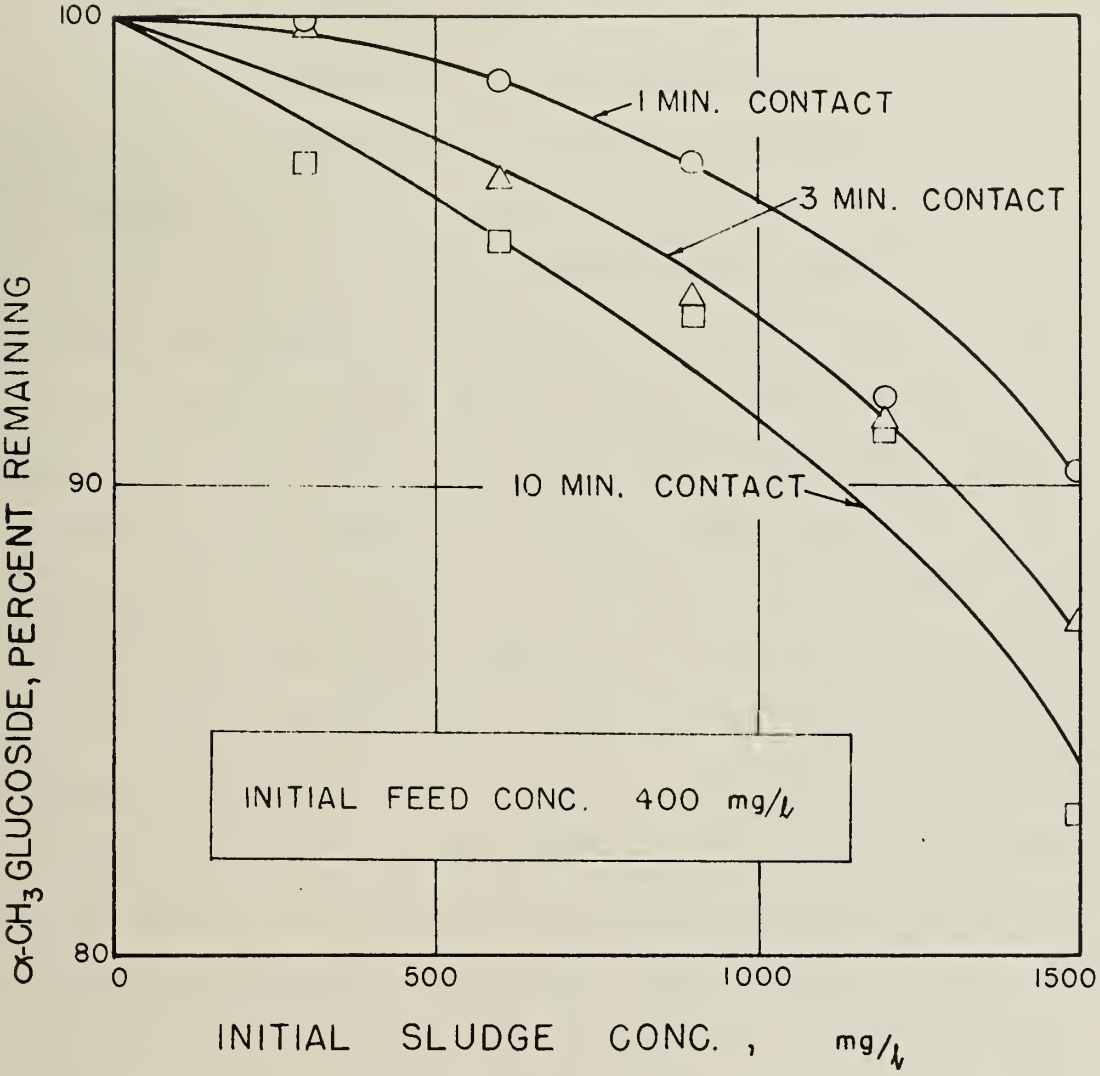

FIGURE 26. INITIAL REMOVAL OF α-METHYL GLUCOSIDE BY DIFFERENT CONC. OF GLUCOSE ACCLIMATED SLUDGE SOLIDS

1. Effect of stabilization of sludge on selection of population

Since it is postulzted that the decrease in activity of sludge organisms, with increasing stabilization periods, is due to the inactivation of inducible enzyme systems, it was thought to be necessary first to establish the effect of stabilization on the distribution of organisms having constitutive and inducible enzyme systems. According to Gauses theorem, when organisms of the same trophic level are competing for the same food, the one whose growth rate is highest should predominate, eliminating all competing organisms. In the contact stabilization process, the fraction of the population which requires inducible enzyme systems for metabolizing a particular waste loses these enzymes during stabilization when it is aerated in the absence of substrate. These organisms are then at a disadvantage when again brought in contact with incoming waste. During the initial period of contact their growth rate is limited by the rate of cynthesis of inducible enzyme systems. Therefore such organisms should be eliminated from the system.

Figure 27 shows the lactose removal capacity of different sludges acclimated to lactose, as a function of stabilization time. The young sludge had never been exposed to starvation. These organisms were harvested from the parent unit as soon as they had exhausted the substrate and had entered the stationary phase of growth (Figure 8). The other sludges were obtained on different days from the fill and draw unit, seeded from the young cells. Organisms from this unit were subjected to periods of starvation (Figure 9), after which a portion of the population was wasted and the unit was fed again. In this manner the organisms which had constitutive enzymes for the utilization of lactose had the advantage and could predominate. This process of selection was similar to that of a contact

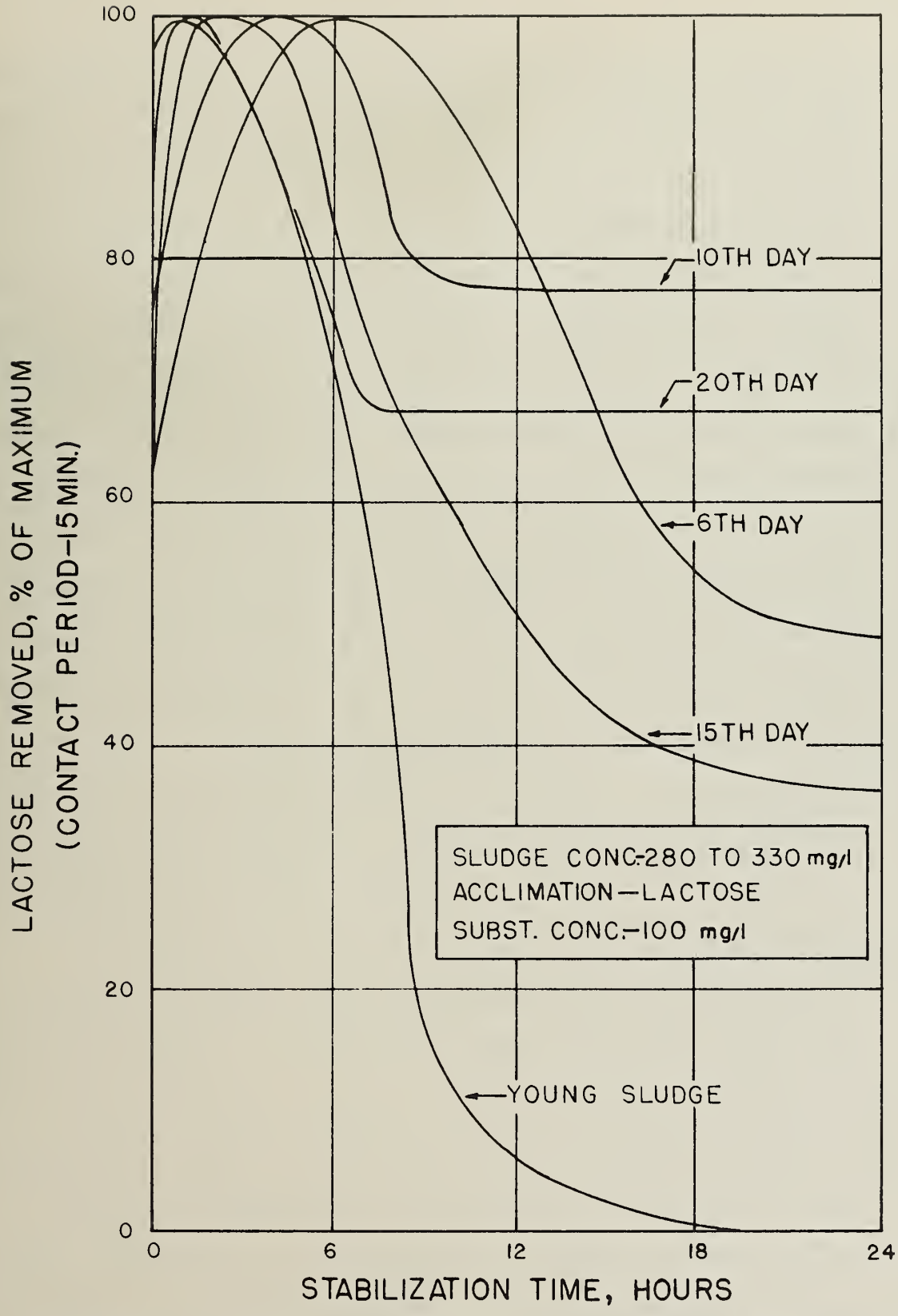

FIGURE 27. SELECTION OF ORGANISMS HAVING CONSTITUTIVE ENZYMES WITH TIME OF OPERATION OF THE ACTIVATED SLUDGE UNIT

stabilization system. In Figure 27 the substrate removal capacity has been expressed as percent of the maximum removal. It is seen that for young cells, after 10 hrs of stabilization the organisms retained only 10 percent of their maximum activity. Stabilization periods greater than 20 hrs resulted in a complete loss of the capacity of the organism to remove lactose. The other sludges also behaved in a similar manner. However, they did not lose their activity completely. The activity of the 10th and 20th day sludges leveled off and remained constant at 77 and 67 percent of the maximum, respectively, after about 10 hrs. The decrease in activity of 6th and 15th day sludges continued for a longer period of time. They retained only 49 and 36 percent of the maximum activity, respectively, at the end of the stabilization period. If it is assumed that the decreased activity of the organisms is due to the loss of the inducible enzymes, the levels at which the curves became parallel to the x axis reflects the percentage of the constitutive enzymes in the system. It is seen that the young cells sludge did not have a measurable fraction of constitutive enzyme responsible for lactose removal.

An examination of Figure 27 reveals that there is no set pattern of selection of organisms having constitutive enzymes with the days of operation of the unit. Furthermore, in other experiments, young cells grown in the same manner as in this experiment did not lose their activity completely when they were subjected to a 24-hr stabilization period (Figure 31). It appears, therefore, that the population did not shift in any direction completely, i.e., a population of organisms having either all constitutive or all inducible enzymes. This is probably because the selecting force (starvation) is not very rigorous and the adaptive phenomenon (induced synthesis of enzymes) reveals itself too rapidly to allow time

for the selective process to operate. This conclusion is also substantiated by Figure 9. It is seen that there was no measurable lag in the removal of substrate, in the first half hour.

2. Effect of stabilization of sludge on enzyme systems

As mentioned previously, the three enzyme systems involved in the metabolism of lactose are β-galactoside permease, β-galactosidase, and the respiratory and synthesizing enzymes. Glucose removal capacity of the sludge was determined so as to measure the activity of the respiratory and synthesizing enzyme systems during stabilization. Since lactose, upon hydrolysis, yields both glucose and galactose the above reasoning presupposes that organisms which metabolize glucose will also metabolize galactose. This assumption was found to be valid as shown by the data in Figure 28. It is seen that galactose was metabolized by glucose acclimated sludge without any lag. The oxygen consumption continued at a constant rate until all the substrate was exhausted. At this time the oxygen consumption rate became equal to the endogenous respiration rate. The oxygen uptake curve for the metabolism of the same concentration of glucose is also shown in the figure. The total consumption of oxygen for glucose was 30 mg/ℓ greater than for galactose. This difference could have been due to experimental errors.

Assuming the metabolism of glucose to be a measure of the activities of respiratory and synthesizing enzymes also presumes that permeation of glucose is not the rate limiting reaction. From Figure 13 it is seen that permeation of glucose was not the rate limiting reaction.

Figure 29 shows the capacity of young lactose acclimated cells to remove glucose and lactose as a function of stabilization time. The

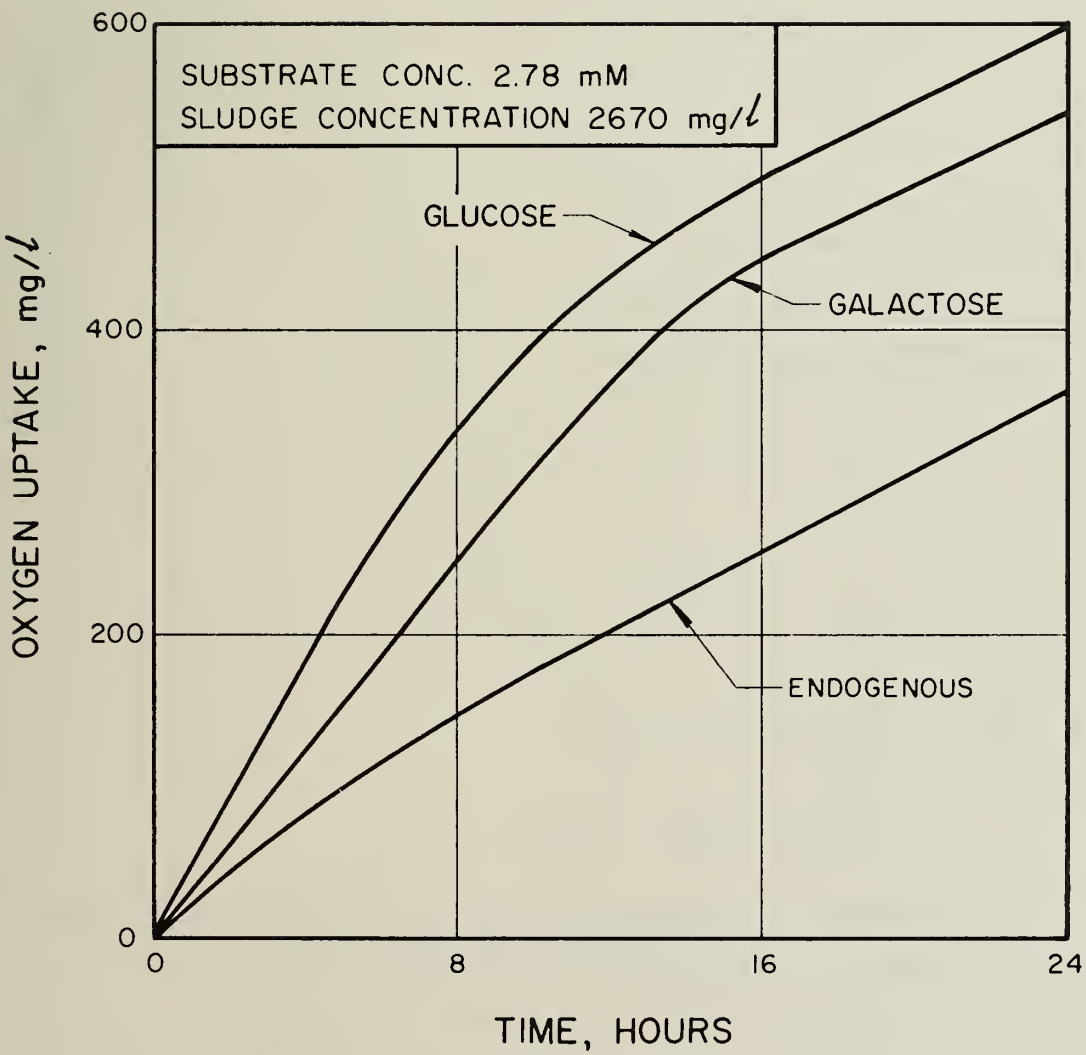

FIGURE 28 OXIDATION OF EQUIMOLAR SOLUTIONS OF GLUCOSE AND GALACTOSE BY GLUCOSE ACCLIMATED SLUDGE

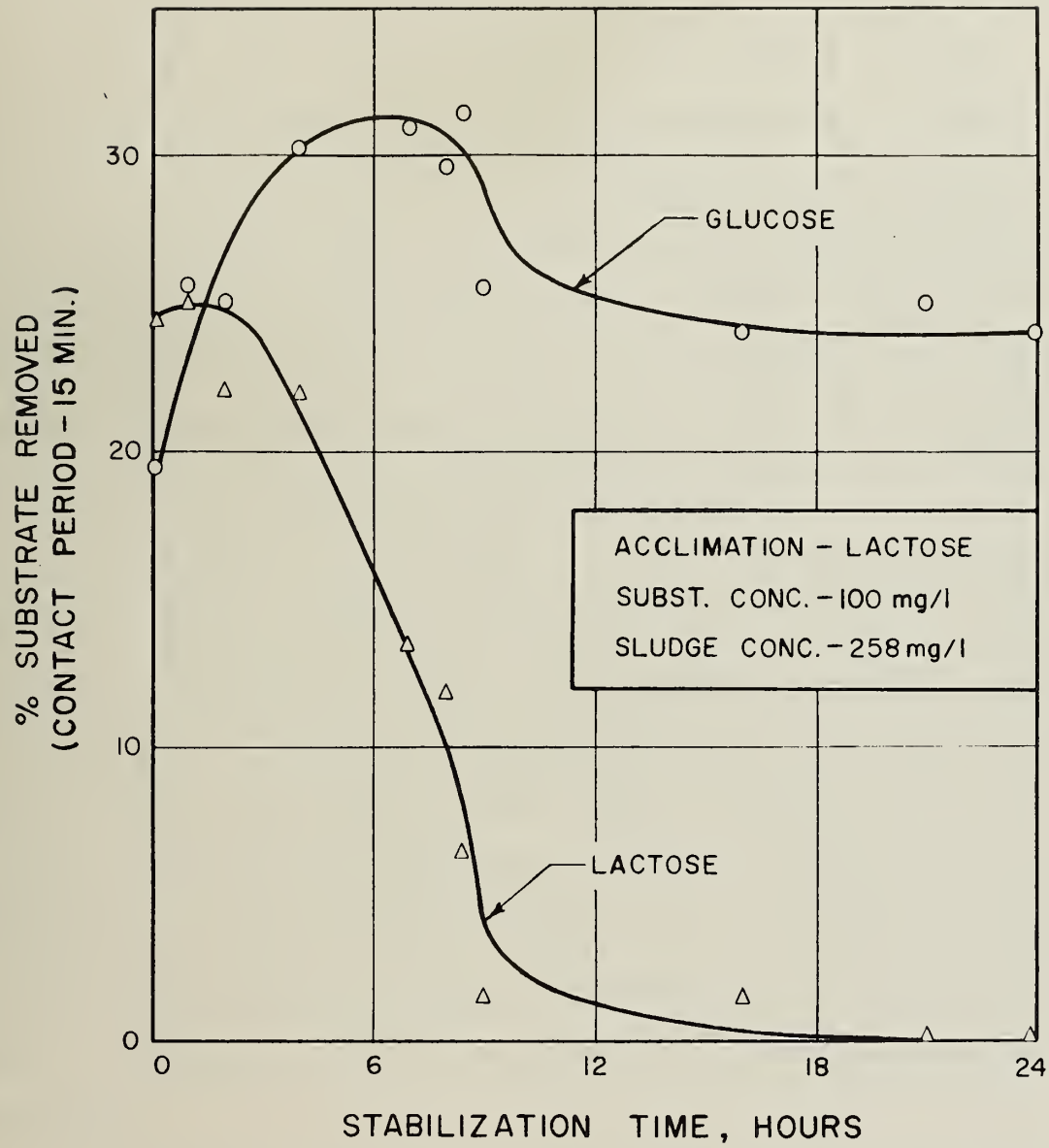

FIGURE 29. SUBSTRATE REMOVAL CAPACITY OF LACTOSE ACCLIMATED YOUNG CELLS AS A FUNCTION OF STABILIZATION TIME

same two relationships are also shown in Figure 30 for the 15th day sludge. While the young sludge lost its capacity to remove lactose completely after 20 hrs of stabilization and the 15th day sludge lost 64 percent of its maximum activity in 24 hrs, the loss of capacity of these sludges to remove glucose was much less. The young and 15th day sludges retained 75 and 93 percent of their maximum glucose removal capacity, respectively. In the young cells the decrease in glucose activity occurred after about 8 hrs of stabilization while in the 15th day sludge the fall in activity was towards the end of the 24-hr stabilization period.

The ability of the organisms to retain their synthesizing and respiratory enzymes for longer periods of time and at a high level could be due to the following two reasons. Since the basic reactions of synthesis and respiration are common to the utilization of all substrates, these enzymes are synthesized constitutively. Secondly, substrates for the reactions of synthesis and respiration are produced internally also, just as endogenous cellular metabolism continues even in the absence of exogenous substrate. Therefore, this provides an opportunity for even the inducible enzymes of the system to survive long after the extracellular substrate is exhausted.

The other two enzyme systems, β-galactosidase and β-galactoside permease were studied by measuring the hydrolysis of o-nitrophenyl galactoside (ONPG) by intact and lysed cells. Figure 30 also shows the β-galactosidase activity of intact cells. It is seen that the variation of the lactose removal parallels that of β-galactosidase activity. The results of a similar experiment with young cells are shown in Figure 31. The results are plotted as percent of the maximum activity. Both these experiments indicate that the decrease in lactose activity can be accounted for

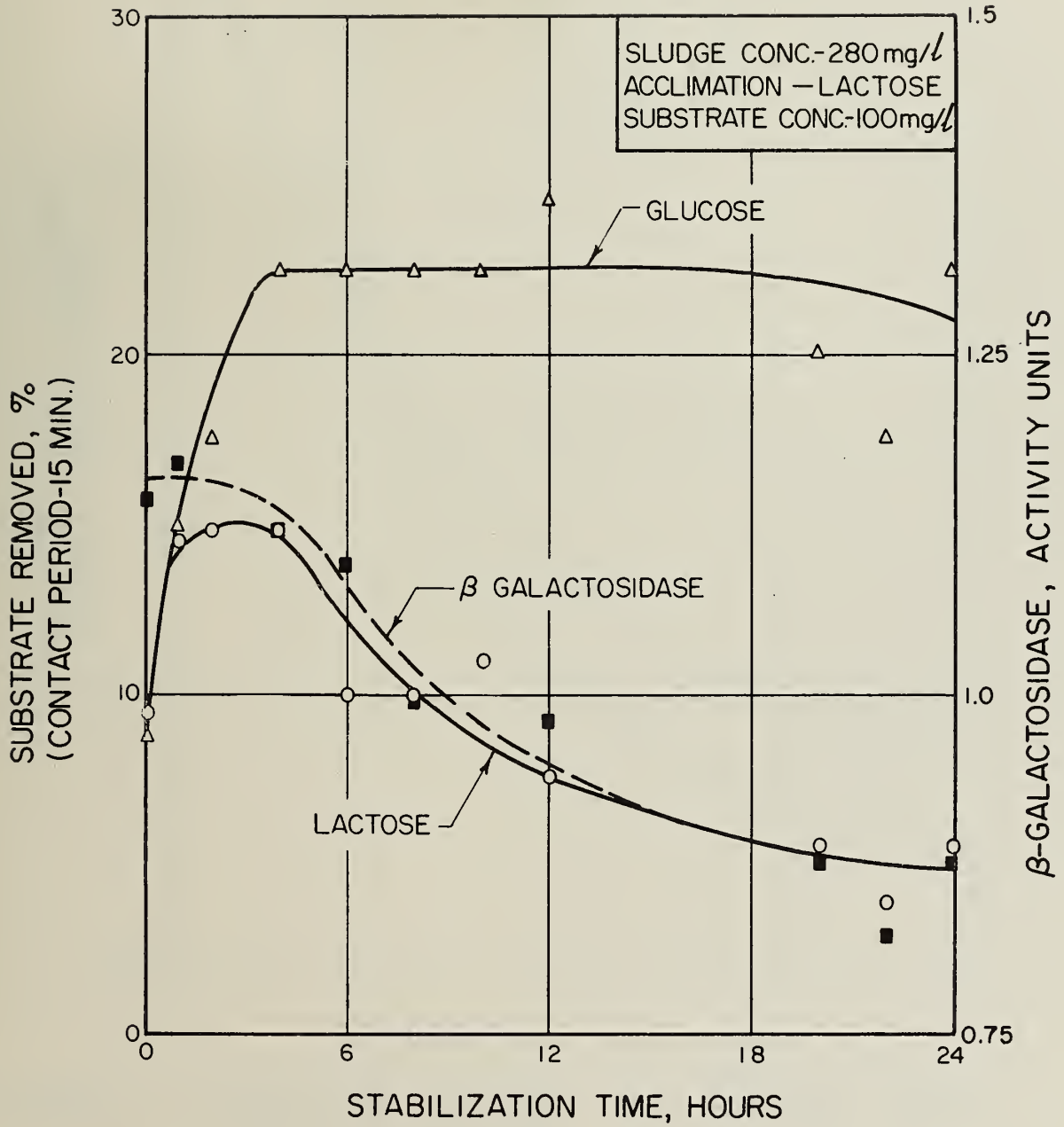

FIGURE 30. SUBSTRATE REMOVAL CAPACITY AND β-GALACTOSIDASE ACTIVITY OF 15th DAY SLUDGE AS FUNCTIONS OF STABILIZATION TIME

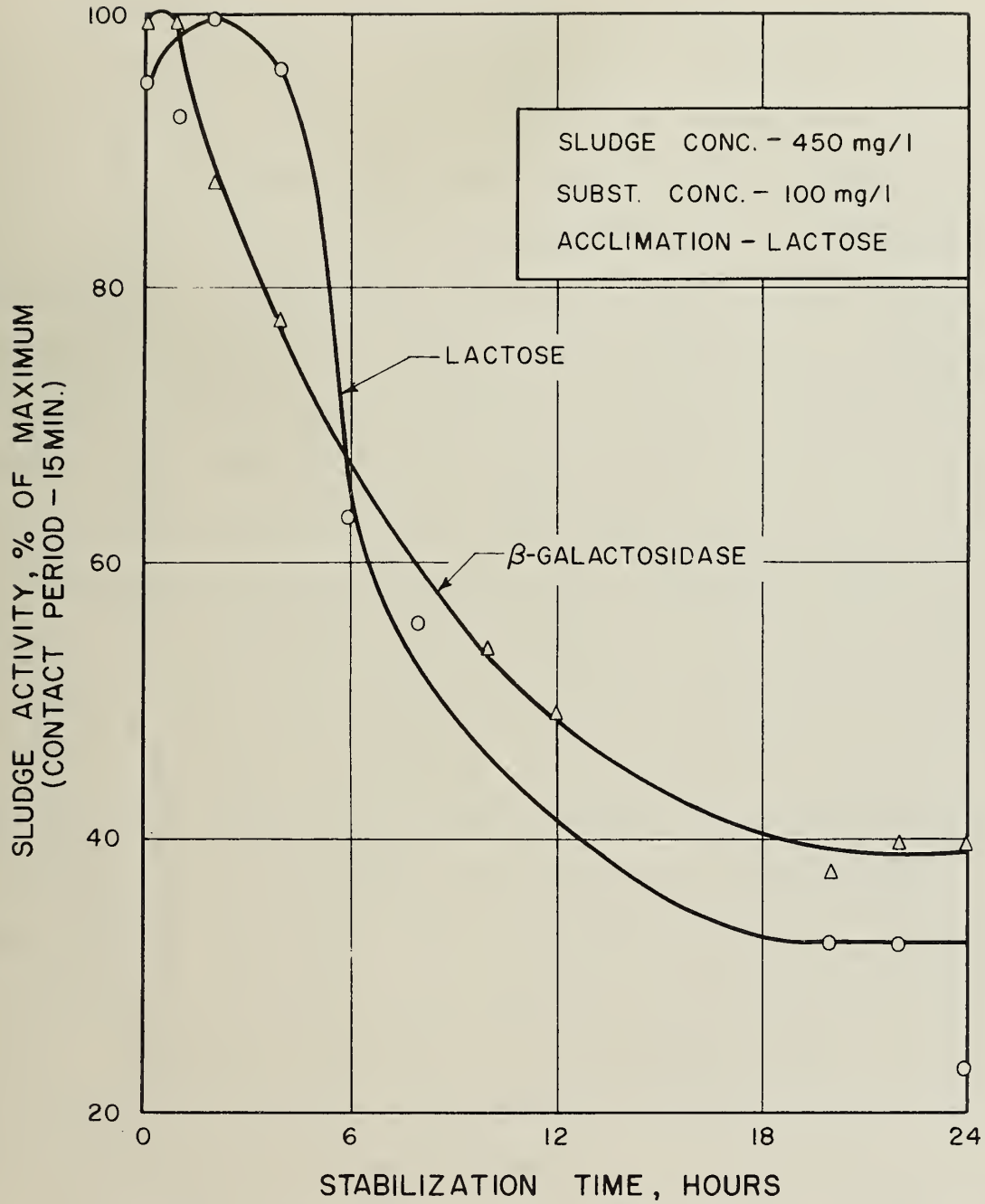

FIGURE 31. PERCENT CHANGE IN LACTOSE AND β-GALACTOSIDASE ACTIVITIES OF LACTOSE ACCLIMATED YOUNG CELLS AS FUNCTIONS OF STABILIZATION TIME

by the decrease in β-galactosidase activity. It should be noted that before ONPG is hydrolyzed by β-galactosidase it is transported by the β-galactoside permease into the cell. Therefore, if permeation is the rate limiting step in the hydrolysis of ONPG, the rate of *in vivo* hydrolysis of ONPG represents the activity of the permease system. To investigate this possibility, β-galactosidase activity of intact cells and of cells after lysis with toluene was determined during stabilization. The results of this experiment are shown in Figure 32. Throughout the stabilization period the cells after lysis showed a higher activity. The ratio of the β-galactosidase activity of the cells after lysis to the activity of intact cells increased from 6 at the beginning of the stabilization period to 18 at the end. This was due to a greater decrease in the activity of the intact cells. This is also revealed by the plot of Figure 33, which shows the activity as percent of the initial value. While the activity of the cells after lysis decreased by 50 percent during 24 hrs, there was almost a 90 percent loss in the activity of the intact cells during the same period. Therefore the higher activity of the cells after lysis indicates that utilization of lactose by the organisms is controlled by the rate at which it is transported inside the cell by the permease system. Further, the decrease in the capacity of the organisms to utilize lactose was due to the inactivation of the permease system.

Thiomethyl-β-galactoside (TMG), an analogue of lactose, is known to be an inducer of β-galactoside permease and β-galactosidase. The compound itself is not hydrolyzed by β-galactosidase of *E. coli* and therefore does not serve as a source of carbon and energy for this organism. Therefore it was decided to determine the effect of TMG on the activities of β-galactoside permease and β-galactosidase during stabilization. However, it

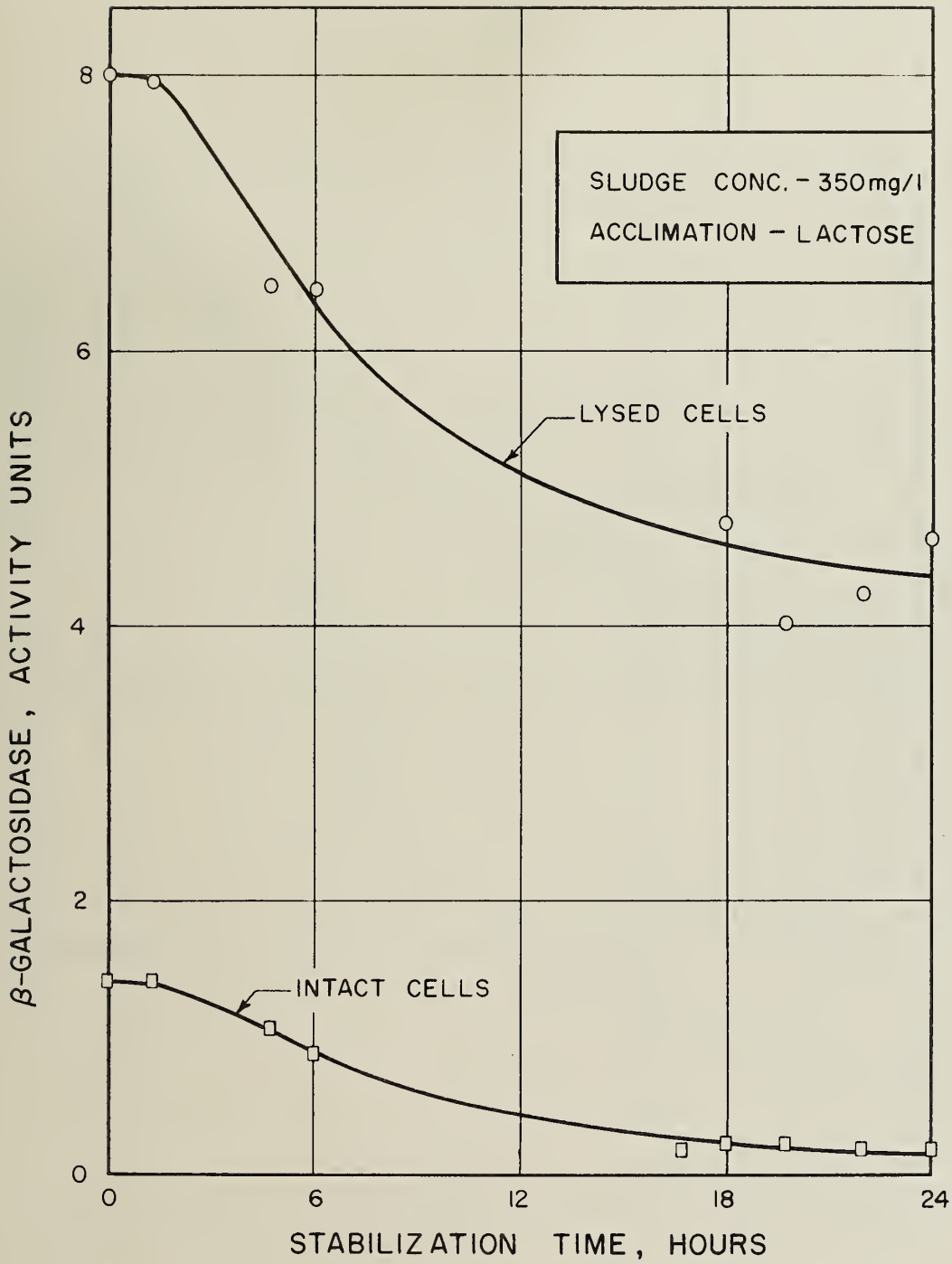

FIGURE 32. β-GALACTOSIDASE ACTIVITY OF LYSED AND INTACT YOUNG CELLS AS A FUNCTION OF STABILIZATION TIME

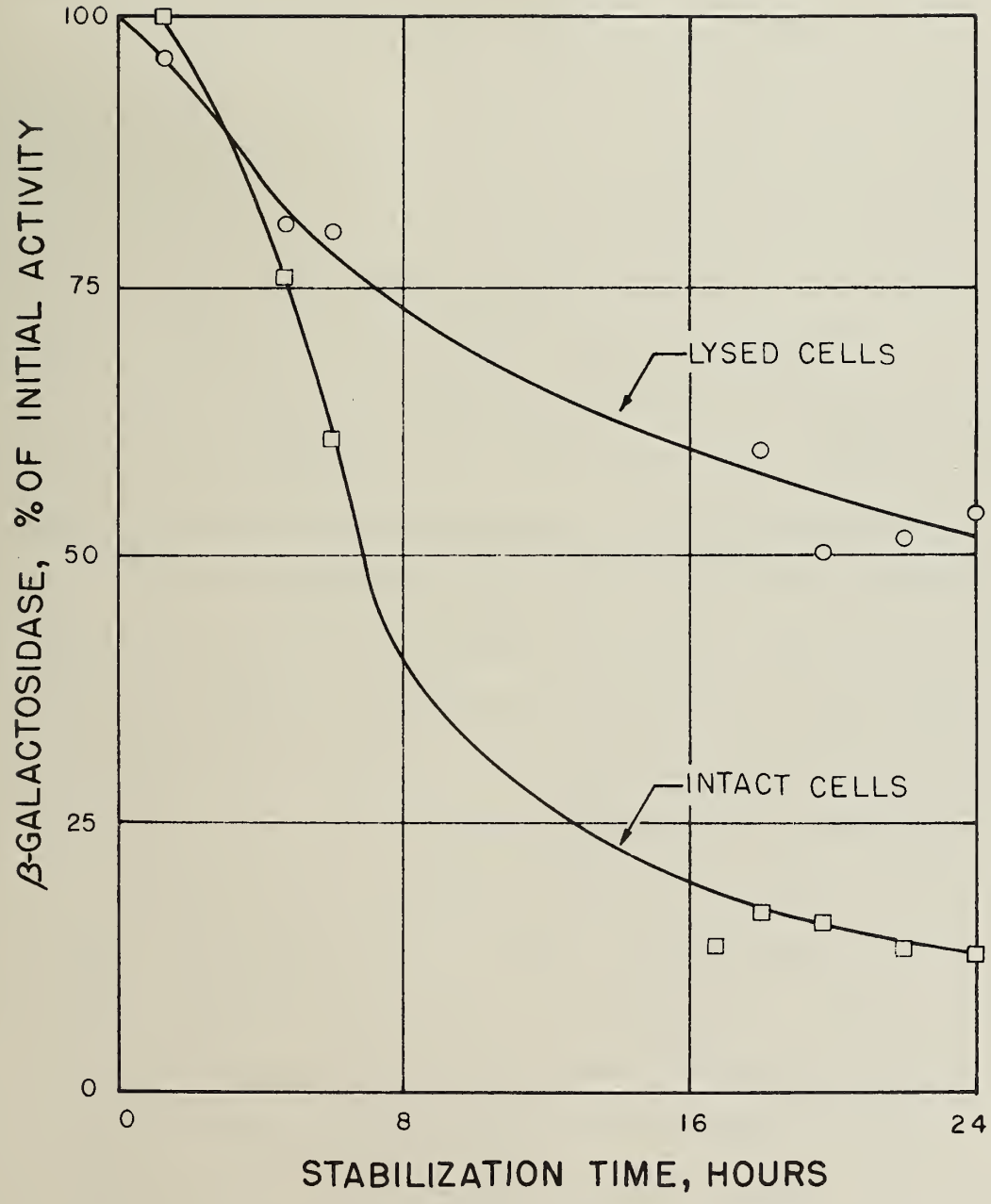

FIGURE 33. PERCENT CHANGE IN β-GALACTOSIDASE ACTIVITY OF LYSED AND INTACT CELLS AS A FUNCTION OF STABILIZATION TIME

was necessary to establish the response of the organisms under study to this compound. Figure 34 shows the oxygen uptake by lactose acclimated young cells in the presence of 500 mg/ℓ, as COD, of lactose and TMG and in the absence of any substrate. It is seen that the organisms in the presence of TMG respired at a slightly higher rate as compared to the endogenous respiration rate. This could be due to the energy required to concentrate the substrate inside the cell. It is also possible that it reflects the presence of a small number of organisms which were able to degrade TMG. Comparing the oxygen uptakes in the presence of lactose and TMG it can be concluded that TMG was not completely degraded.

Figure 35 shows the results of an experiment in which the sludge was stabilized in the presence of 5×10^{-4} M TMG. For a comparison the observations of Figure 32 are also plotted. It is seen that, in the presence of TMG, the activities of both intact cells and cells after lysis were maintained at their initial levels for the first 10 hrs. This stabilizing effect of TMG on the ß-galactosidase activity of intact cells further substantiates the presence of an enzyme catalyzed transport system. It is difficult to conceive of a physical phenomenon, adsorption or diffusion, which will satisfactorily explain these observations. The induction of an enzyme, or conversely as in this case, the maintenance of an inducible enzyme, requires the presence of an inducer and a source of energy. It has been shown that bacteria can synthesize enzymes in the absence of an extracellular carbon and energy source by improvising from their storage materials (80). In the present experiment, probably there was sufficient intracellular material to contribute towards synthesis and repair of the degraded enzymes for the first 10 hrs, or the enzymes were not degraded at all because of the presence of their inducer. However,

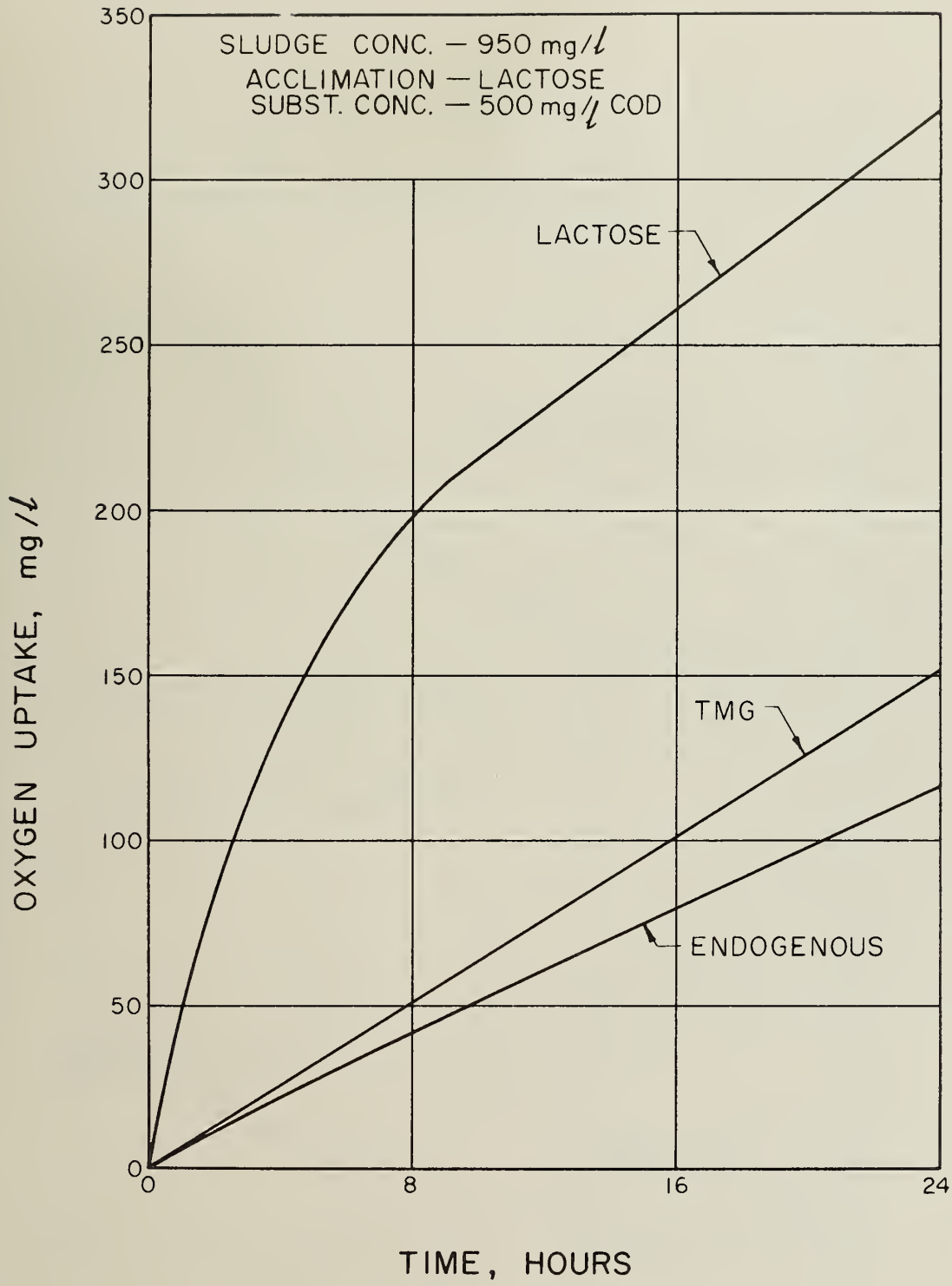

FIGURE 34 OXIDATION OF LACTOSE AND TMG BY LACTOSE ACCLIMATED YOUNG CELLS.

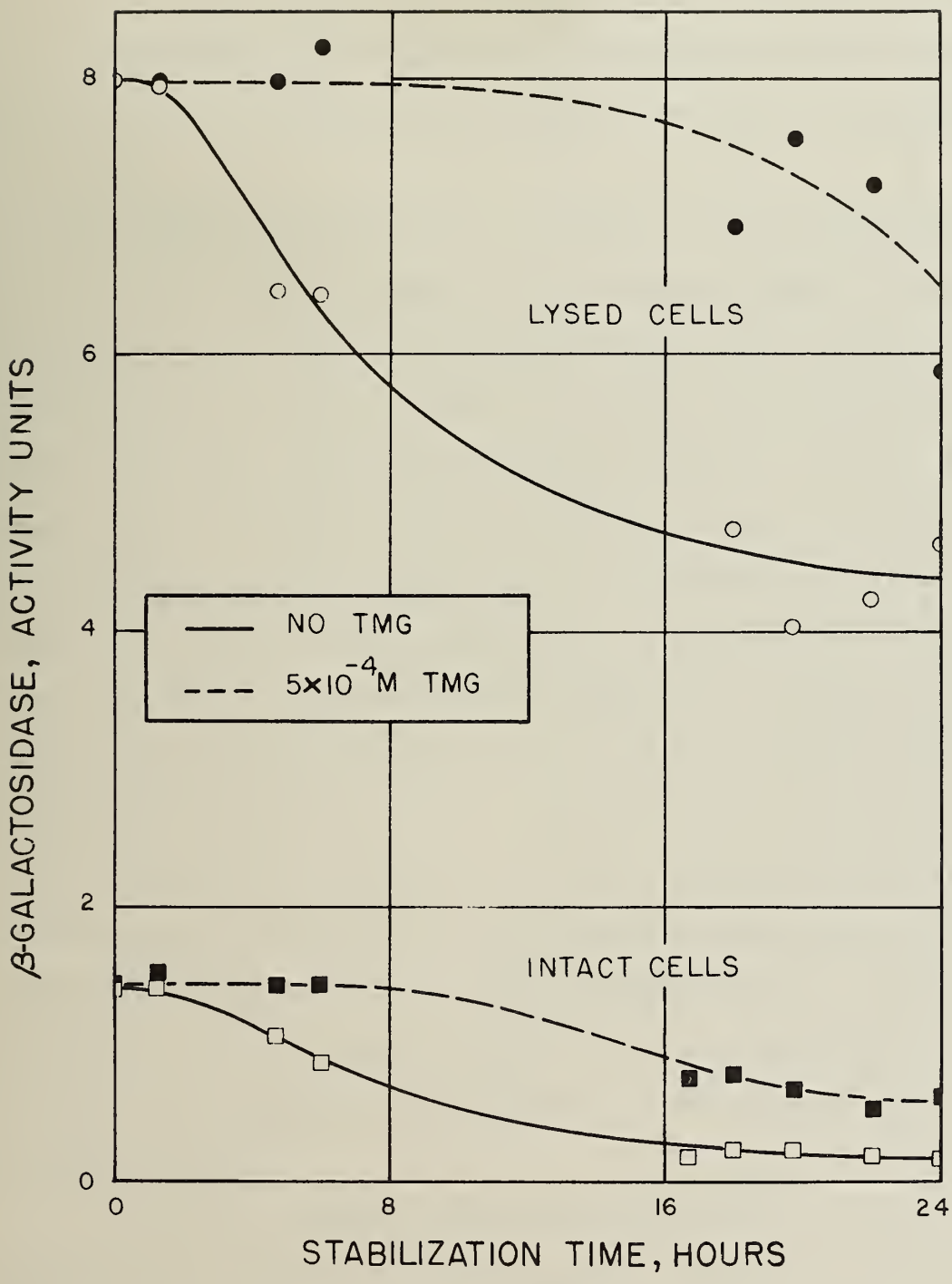

FIGURE 35. EFFECT OF 5×10^{-4} M TMG ON β-GALACTOSIDASE ACTIVITY DURING STABILIZATION OF LACTOSE ACCLIMATED SLUDGE

once the endogenous reserve was exhausted, the presence of the inducer by itself could not preserve the enzymes.

The above analysis does not preclude the possibility of a system in which the permease will not be the limiting enzyme. In such a system the activity of the sludge will be governed by the rate limiting enzyme.

3. Effect of stabilization of sludge on cellular constituents

Even though the inactivation of inducible enzymes accounts for the decrease in the sludge substrate removal rates, with extended periods of stabilization, it does not explain the initial increase in the activity of the sludge during stabilization. Figure 27 shows that there is an increase in the activity of different sludges with time of stabilization up to the first six hours. The variation in the behavior of different sludges is attributed to the inconsistency in the preparation of sludges before initiation of stabilization. The preparation involved an initial contact period with the substrate at room temperature followed by two washings of the sludge at 3 to 5° C. It was not always possible to rigidly follow the same time and temperature conditions during these operations. These conditions are believed to be significant in controlling the behavior of the sludge during the first few hours of stabilization. With one exception it can be concluded from Figure 27 that the sludges having lower activity at the beginning of the stabilization period took longer to attain the maximum activity.

In order to delineate these observations, it was decided to determine if the changes in different cellular constituents during stabilization could be related to sludge activity. Figure 36 shows the changes in various system parameters during stabilization of lactose acclimated,

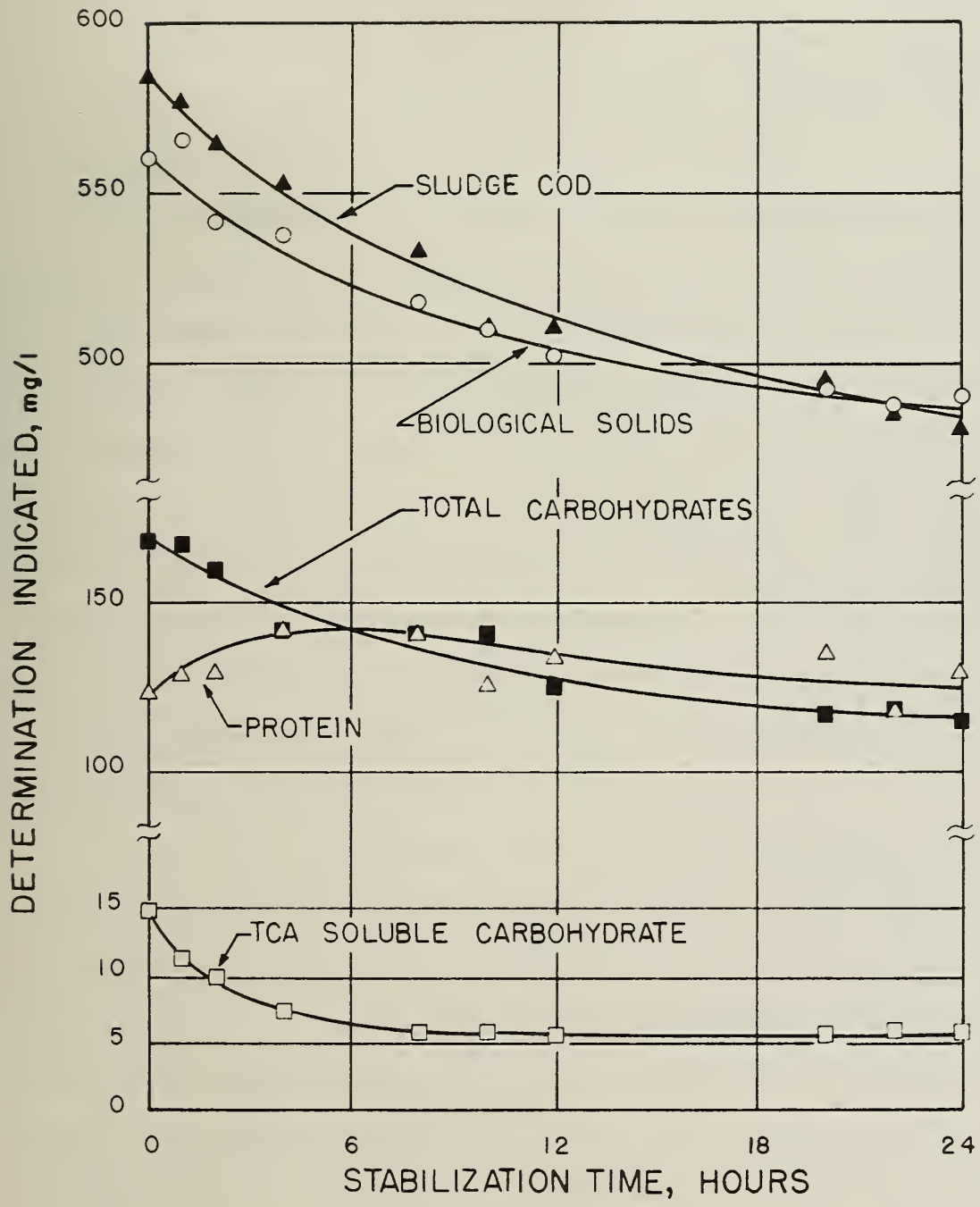

FIGURE 36. CHANGES IN SYSTEM PARAMETERS DURING STABILIZATION OF LACTOSE ACCLIMATED 15 DAY SLUDGE

15th day, sludge. The data in Figure 36 are reported as concentrations in the sludge suspension. It is seen that the sludge concentration decreased considerably during the stabilization period. Therefore the curves, shown in Figure 37, were computed to obtain the net change in any particular sludge characteristic above the minimum. For example, the initial total carbohydrate value was obtained as follows.

An examination of the ratios of sludge and carbohydrate concentration, at different times during the stabilization period, shows that the minimum total carbohydrate content of the sludge occurred at 24 hrs. It was equal to $\frac{115}{486}$ mg total carbohydrate/mg sludge. At 0 hr the sludge and carbohydrate concentrations were equal 560 and 170 mg/ℓ, respectively. Therefore the net change above the minimum value was $170 - \frac{115}{486} \times 560 =$ 38 mg/ℓ. The data for these computations were obtained from the curves fitted visually to the observed data in Figure 37. Use of the observed data for computation purposes compounded the errors in the observations and resulted in a wide scatter of the data. Therefore, the use of the observed data had to be abandoned.

It is seen from Figure 37 that the total carbohydrate content of the freshly fed sludge decreased sharply for the first eight hours. This was accompanied by an increase in the protein content. The increase in protein content at the expense of cellular carbohydrates could indicate synthesis of enzymes from the carbohydrate accumulated during the initial contact with lactose. Since the activity of β-galactosidase did not increase at any time during stabilization above the initial value (Figure 30), it appears that these enzymes belong to the system of respiratory and synthesizing enzymes. This also means that while the organisms were able to attain the maximum β-galactosidase activity during the initial contact

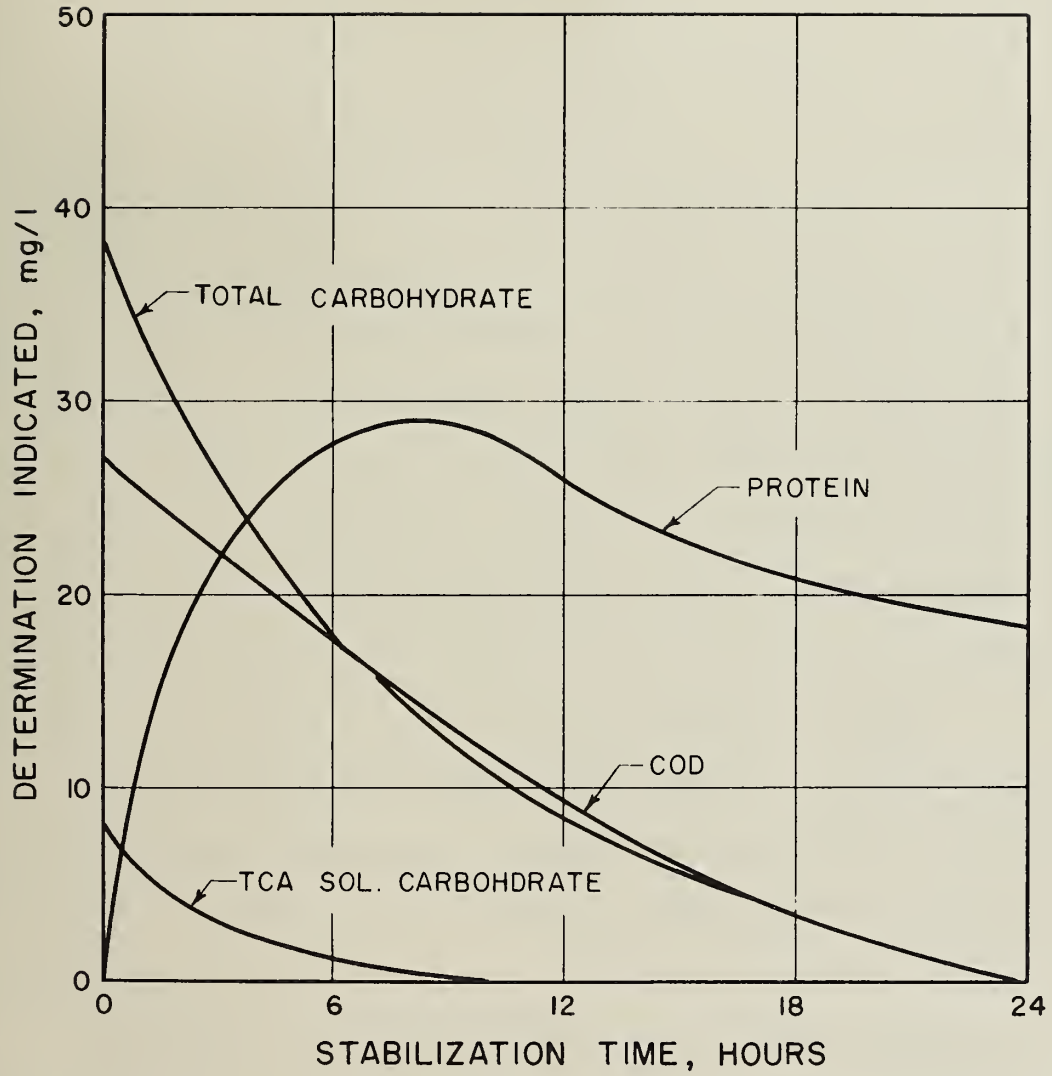

FIGURE 37. NET CHANGES IN SLUDGE CHARACTERISTICS AS A FUNCTION OF STABILIZATION TIME

with lactose, the other enzymes required a longer period of induction. Another factor which may contribute to the activity of organisms during stabilization is the TCA soluble carbohydrate fraction of the cells. It is apparent that the substrate will be removed at a higher rate, by consecutive reversible enzymatic reactions leading to oxidation, if the concentrations of the intermediate products are low. The intermediates in the present system also include storage polymers of carbohydrates as they are ultimately oxidized during stabilization. It is seen from Figure 37 that the TCA soluble carbohydrate fraction, which represents small and large carbohydrate molecules, changed during the first few hours from a maximum to the minimum. A similar response was shown by the total carbohydrate content of the cells which decreased at a higher rate during the first six hours of the stabilization period. It is possible that the sludge organisms contain other materials also which behave in a similar manner as the TCA soluble and the total carbohydrate fractions.

It is also seen from Figure 37 that the COD to weight ratio of the sludge organisms decreased at a constant rate throughout the stabilization period. This reflects the formation of more oxidized products within the cells.

To summarize the above observations, the sludge substrate removal capacity during stabilization, for the most part, appears to depend upon the enzymatic activity of the sludge organisms. During the first few hours of stabilization the rate controlling factor in the substrate removal capacity was the induction of respiratory and synthesizing enzymes. During the later part of the stabilization period the substrate removal capacity was a function of the β-galactosidase activity of the intact cells. Another factor which may have contributed to the sludge

activity was the concentration of intermediate products of metabolism and other cellular constituents. The sludge activity was inversely related to the concentration of these constituents. These constituents were endogenously metabolized by the organisms during the first few hours of stabilization.

4. Effect of stabilization period on the performance of contact stabilization process

The experiments described in this section were carried out using the laboratory contact stabilization unit (Figure 11). The unit was fed a 5:1 mixture of glucose and yeast extract on a batch basis. Since almost all the substrate was removed within the first half hour after each feeding, the sludge underwent stabilization until the next feeding. Thus the stabilization period was changed by changing the frequency of feeding. The COD loading per feeding was equal to 0.4 mg COD/mg sludge solids. The frequency of feeding during this study was increased from 2 to 24 times a day. This resulted in an increase in the loading rate from 0.8 to 9.6 mg COD/mg sludge solids/day.

The unit was maintained on a particular feeding schedule for at least 30 feed cycles. The sludge volume index (SVI) and concentration of biological solids in the mixed liquor was determined during this period. Figures 38 through 42 show these observations. Changes in system parameters during a feed cycle at different loading rates are shown in Figures 43 through 46. The zero hour observations in these figures are calculated values based upon the last observation of the same feed cycle. For example the zero hour carbohydrate concentration in Figure 43 was calculated by multiplying the 12 hour value by 0.833, the theoretical dilution ratio after

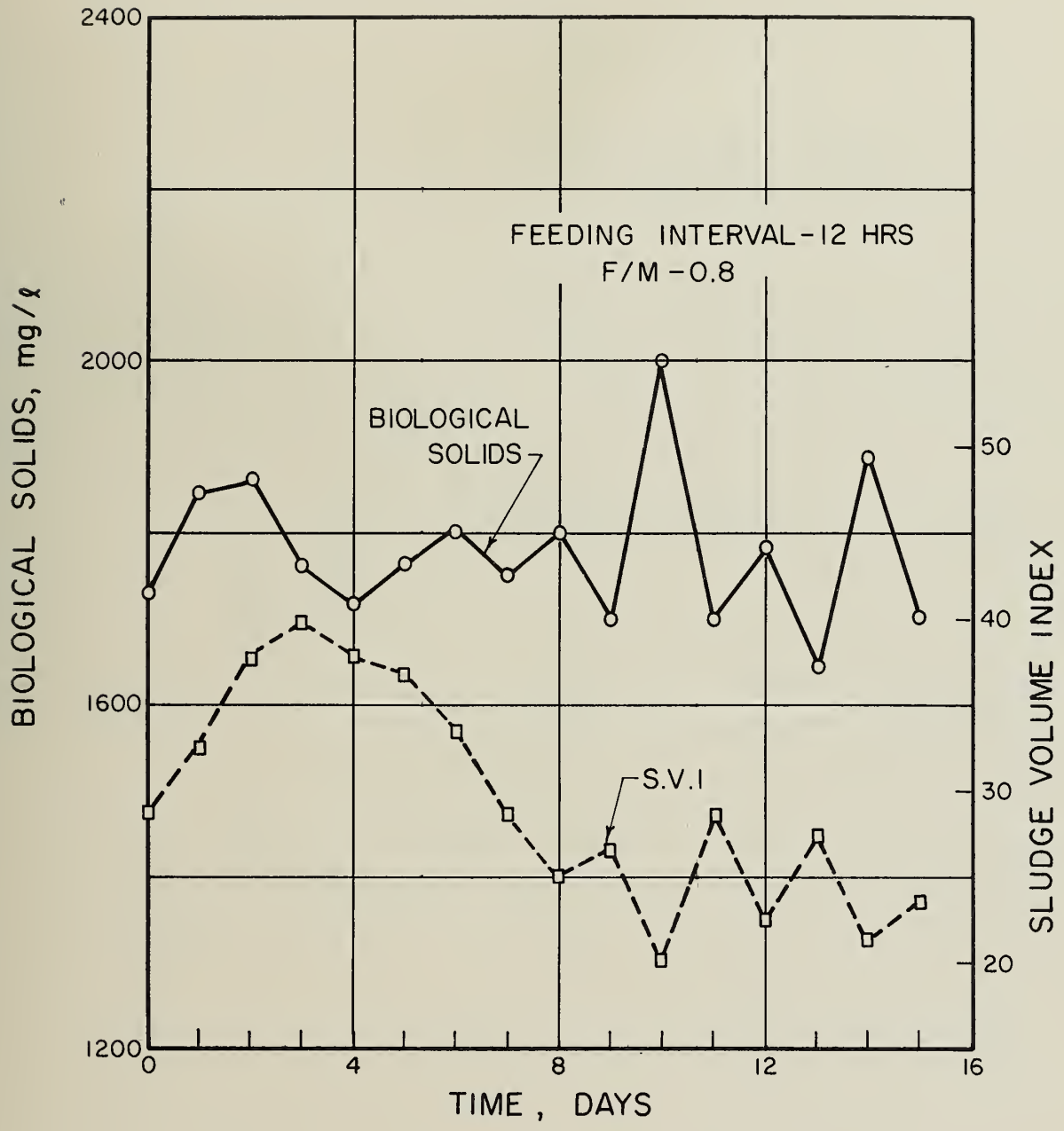

FIGURE 38 BIOLOGICAL SOLIDS CONCENTRATION AND S.V.I. OF MIXED LIQUOR AFTER 12 HRS OF AERATION, F/M 0.8 PER DAY

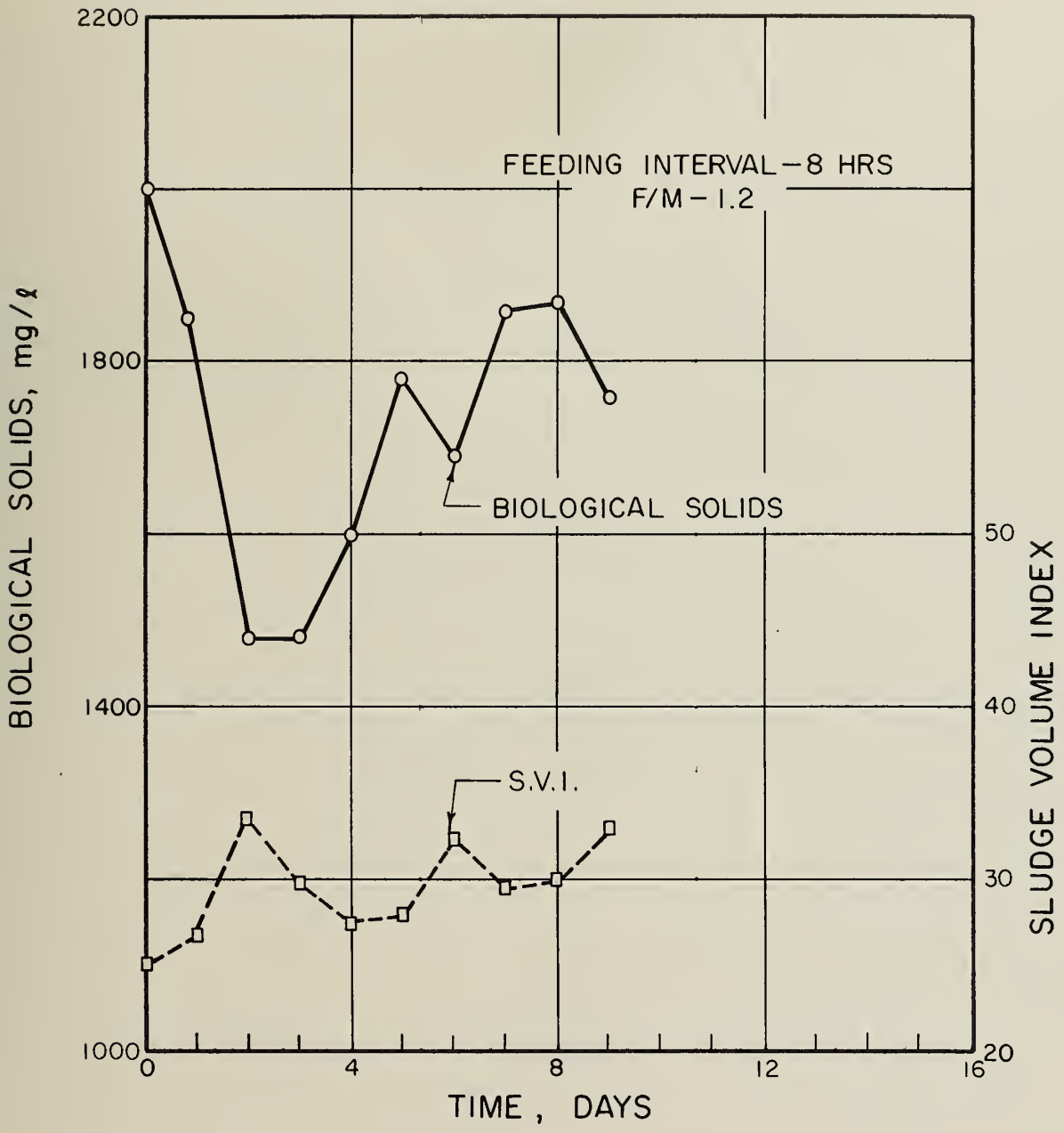

FIGURE 39 BIOLOGICAL SOLIDS CONCENTRATION AND S.V.I. OF MIXED LIQUOR AFTER 8 HRS OF AERATION, F/M 1.2 PER DAY

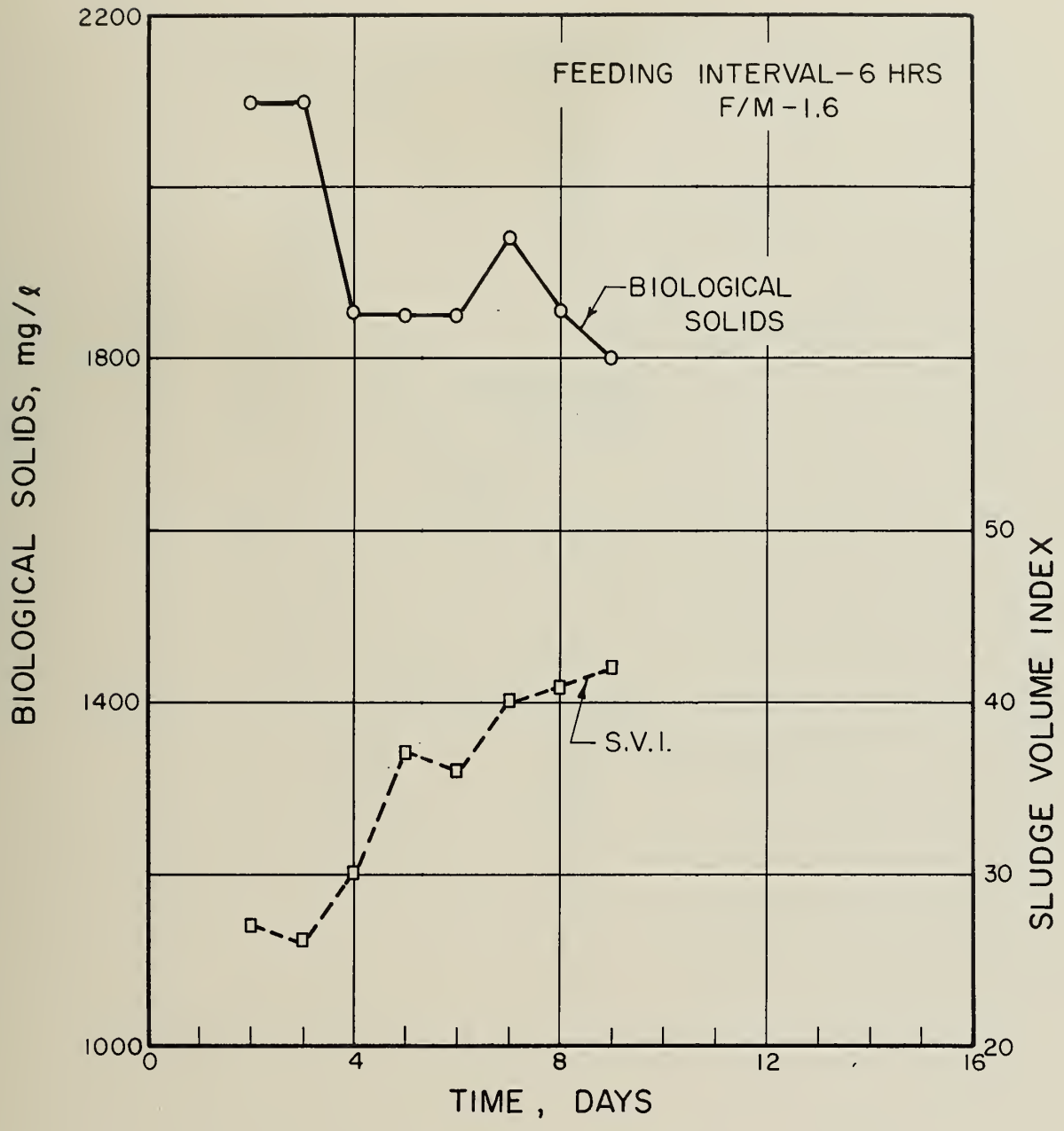

FIGURE 40 BIOLOGICAL SOLIDS CONCENTRATION AND S.V.I. OF MIXED LIQUOR AFTER 6 HRS OF AERATION, F/M 1.6 PER DAY

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University of Illinois
B106 NCEL
208 N. Romine Street
Urbana, Illinois 61801

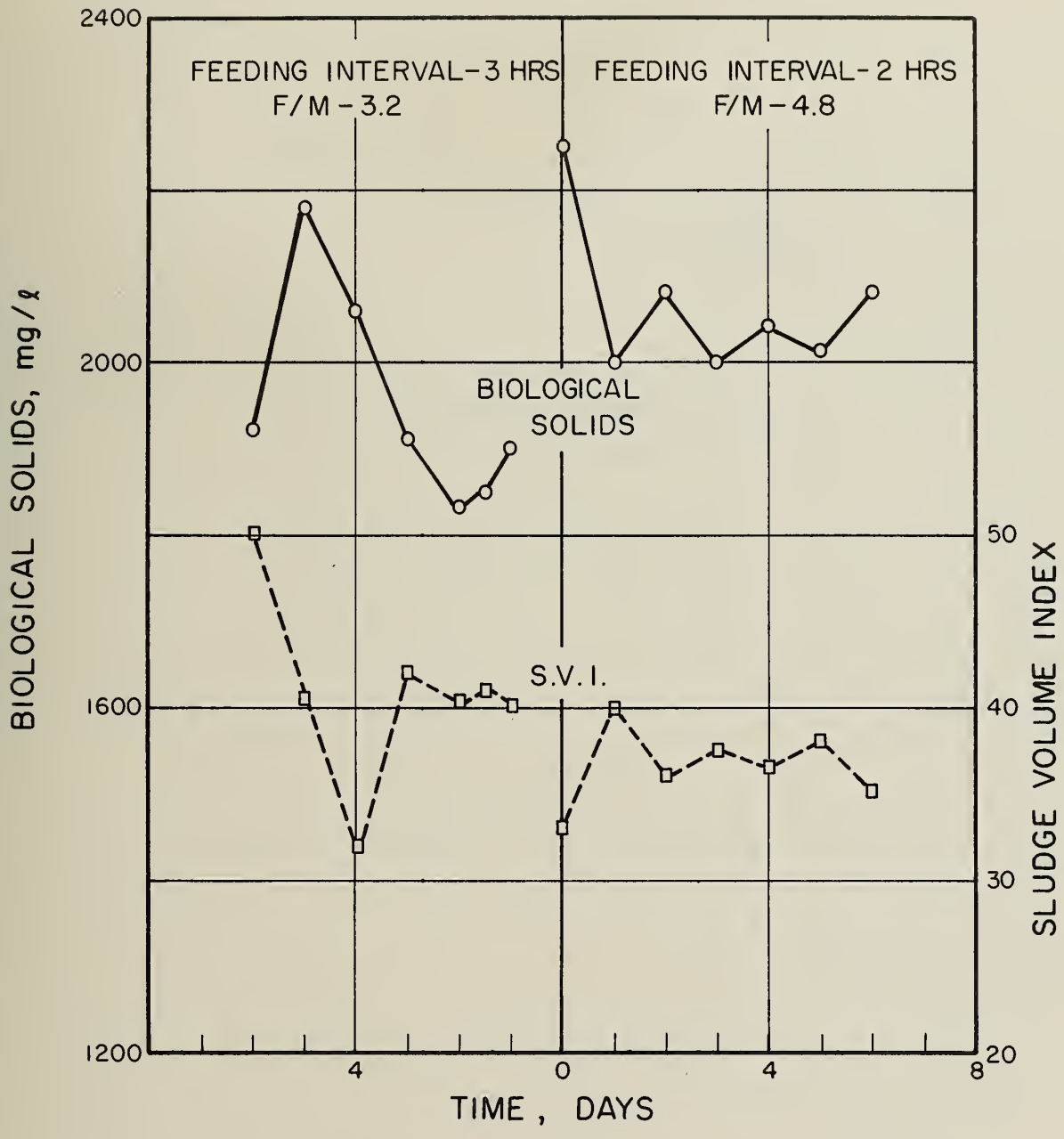

FIGURE 41 BIOLOGICAL SOLIDS CONCENTRATION AND S.V.I. OF MIXED LIQUOR AFTER 3 HRS OF AERATION, F/M 3.2 PER DAY, AND AFTER 2 HRS OF AERATION, F/M 4.8 PER DAY

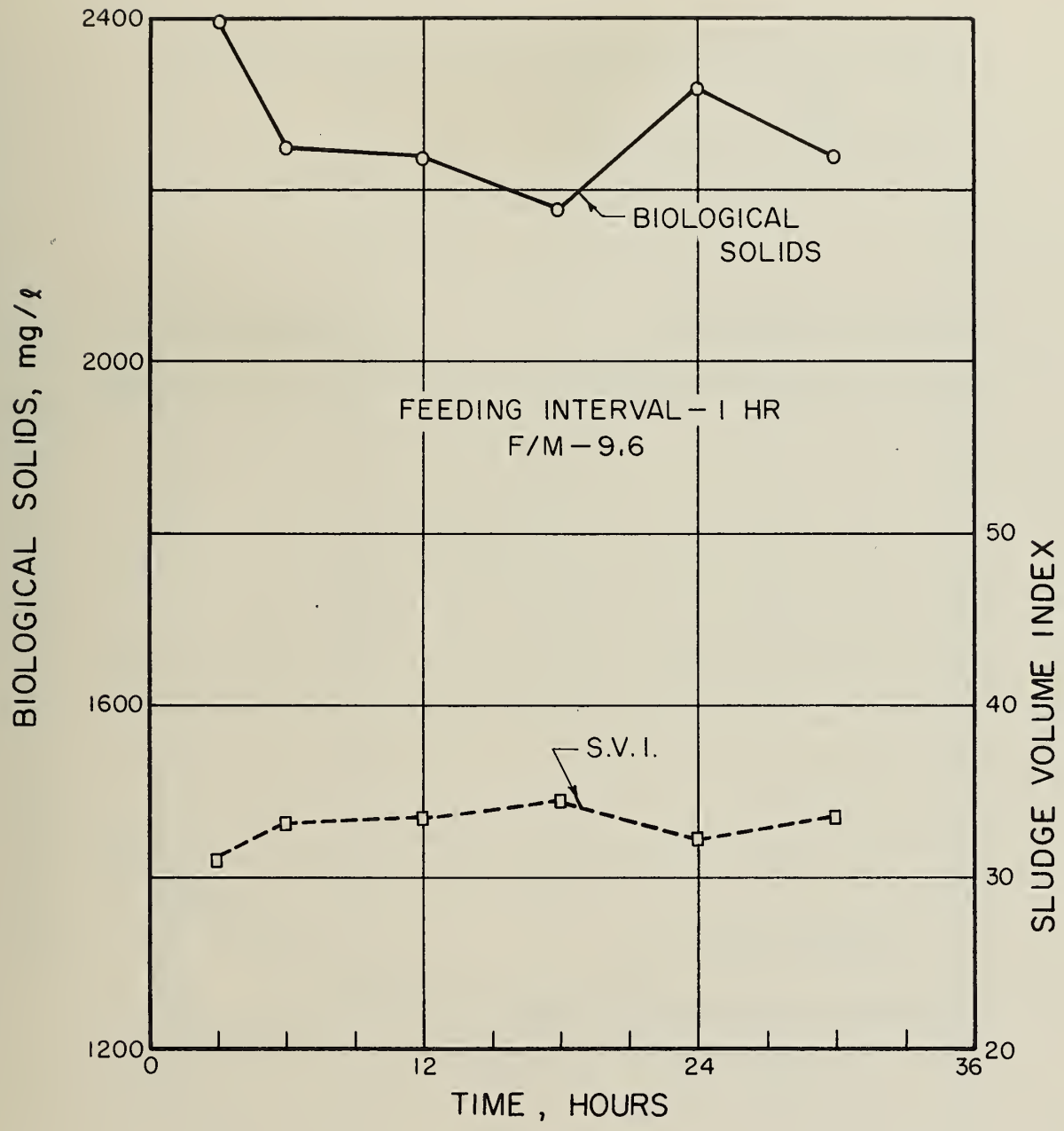

FIGURE 42 BIOLOGICAL SOLIDS CONCENTRATION AND S.V.I. OF MIXED LIQUOR AFTER 1 HR OF AERATION, F/M 9.6 PER DAY

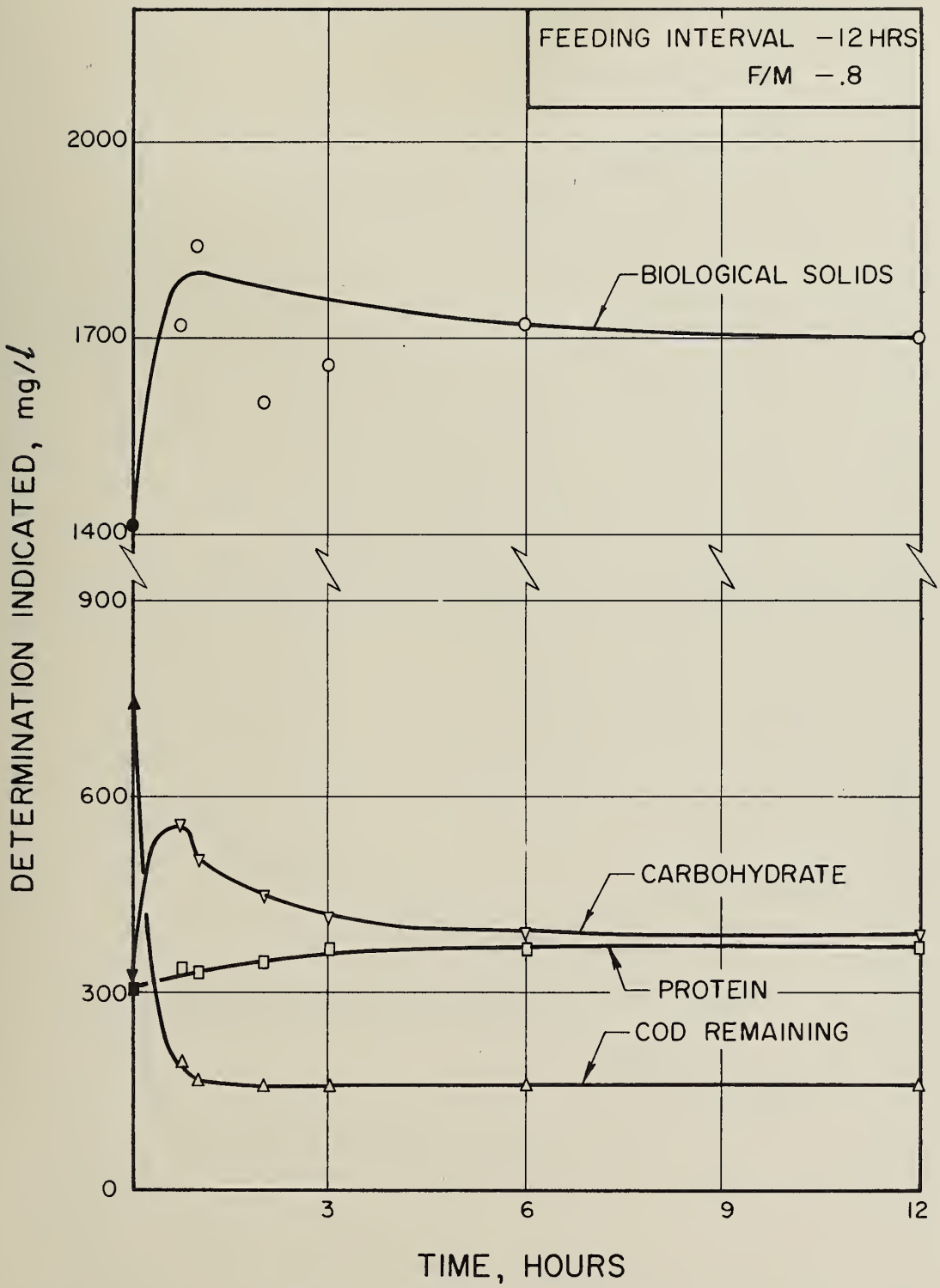

FIGURE 43 CHANGES IN SYSTEM PARAMETERS DURING 12 HR FEEDING CYCLE, F/M 0.8 PER DAY

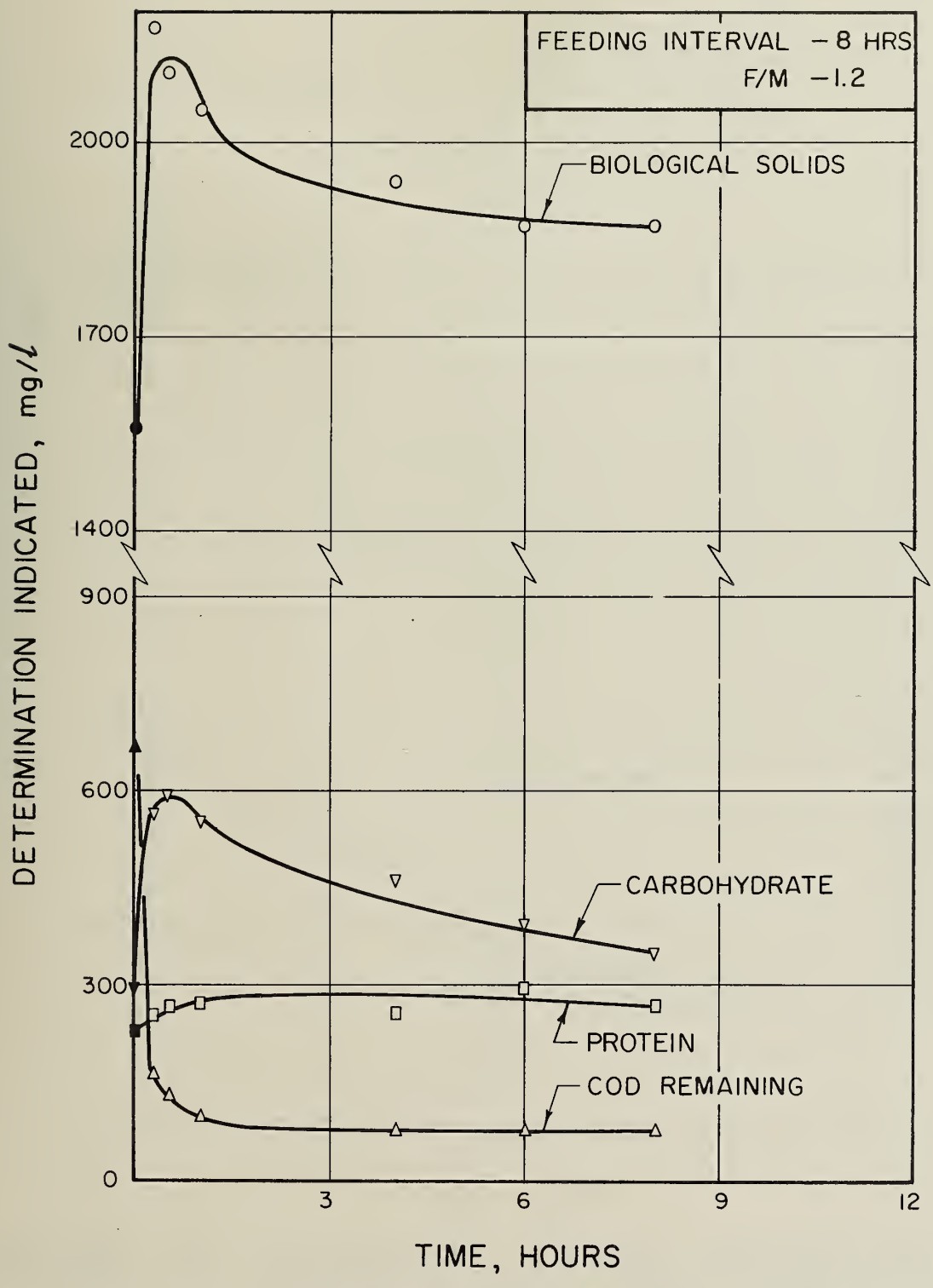

FIGURE 44 CHANGES IN SYSTEM PARAMETERS DURING 8 HR FEEDING CYCLE, F/M 1.2 PER DAY

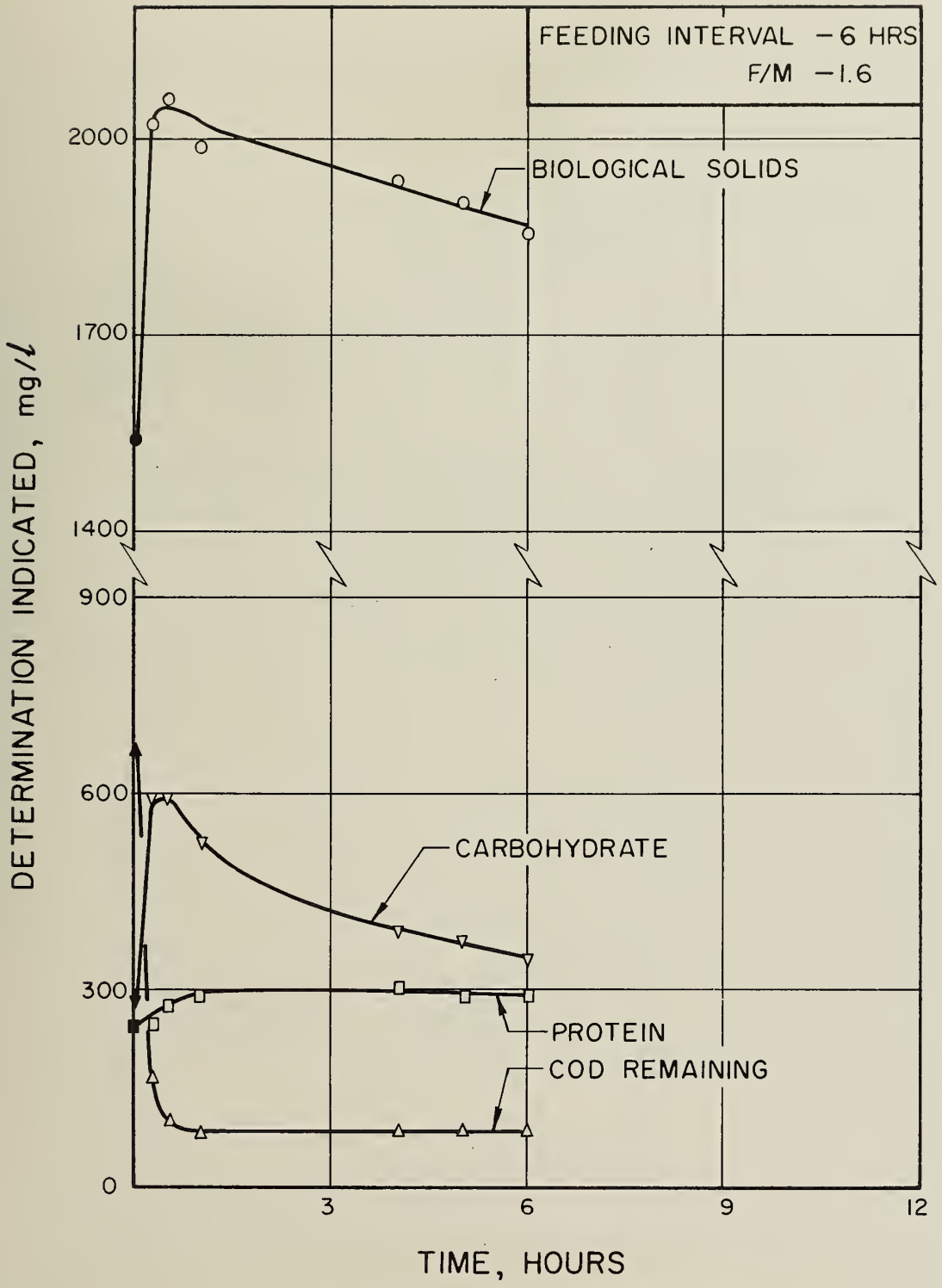

FIGURE 45 CHANGES IN SYSTEM PARAMETERS DURING 6 HR FEEDING CYCLE, F/M 1.6 PER DAY

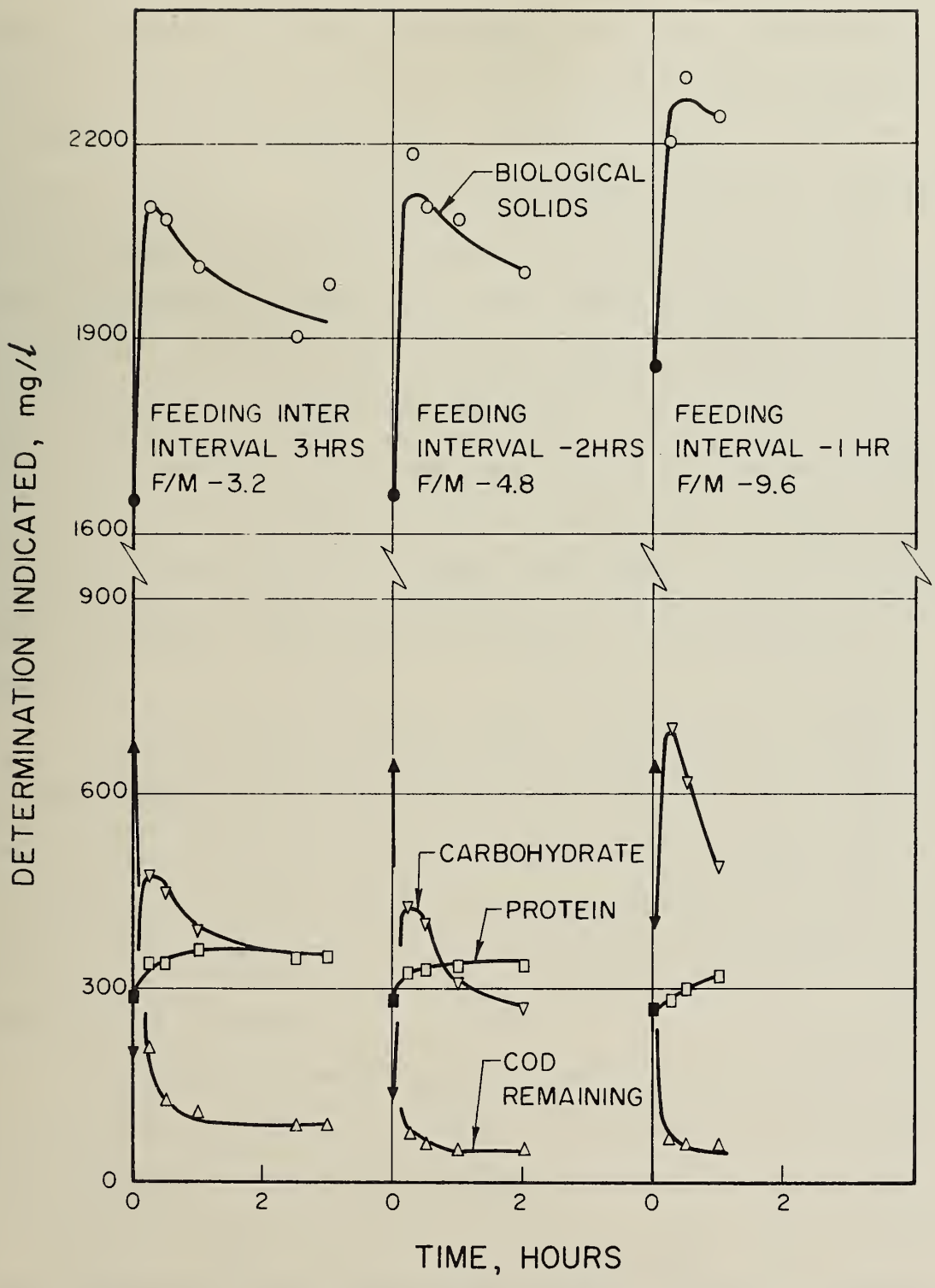

FIGURE 46 CHANGES IN SYSTEM PARAMETERS DURING 3 HR FEEDING CYCLE, F/M 3.2, 2HR FEEDING CYCLE, F/M 4.8, AND 1 HR FEEDING CYCLE, F/M 9.6

each feeding. These operational data and sludge characteristics at different loading rates have been summarized in Figure 47. The equilibrium solids and SVI values at different loading rates have been obtained by averaging the last three observations in the time period as shown in Figures 38 through 42. It is seen that at higher loading rates there was greater net production of sludge solids per unit amount of substrate removed, resulting in a higher equilibrium solids concentration. This can be related to the fact that less time was allowed for stabilization with higher frequencies of feeding. At the highest loading rate of 9.6 mg COD/mg sludge solids/day, the sludge settled leaving a clear supernatant. It had a SVI value lower than the maximum observed for all systems. The maximum SVI value occurred at a loading rate between 1.8 and 3.2 mg COD/mg sludge solids/day. These results are similar to those reported by Stewart (39).

Figures 43 through 46 thow that a certain amount of soluble COD was always present in the system which was not degraded. It is seen from Figure 47 that the system which was stabilized for the longest period of time produced the greatest amount of nondegradable soluble COD. This COD probably results from lysis of cells and secretion of nonmetabolizable by-products by the organisms.

Figure 47 also shows the percent degradable COD removed in the first half hour at different F/M ratios. It is seen that the substrate removal efficiency of the system increased from 89.5 to 98.5 percent as the stabilization time was decreased from 12 to 1 hour. This observation further substantiates the view that during stabilization the enzymes in the organisms are inactivated, which results in a loss of substrate removal capacity of the sludge. There was no increase of sludge activity when the

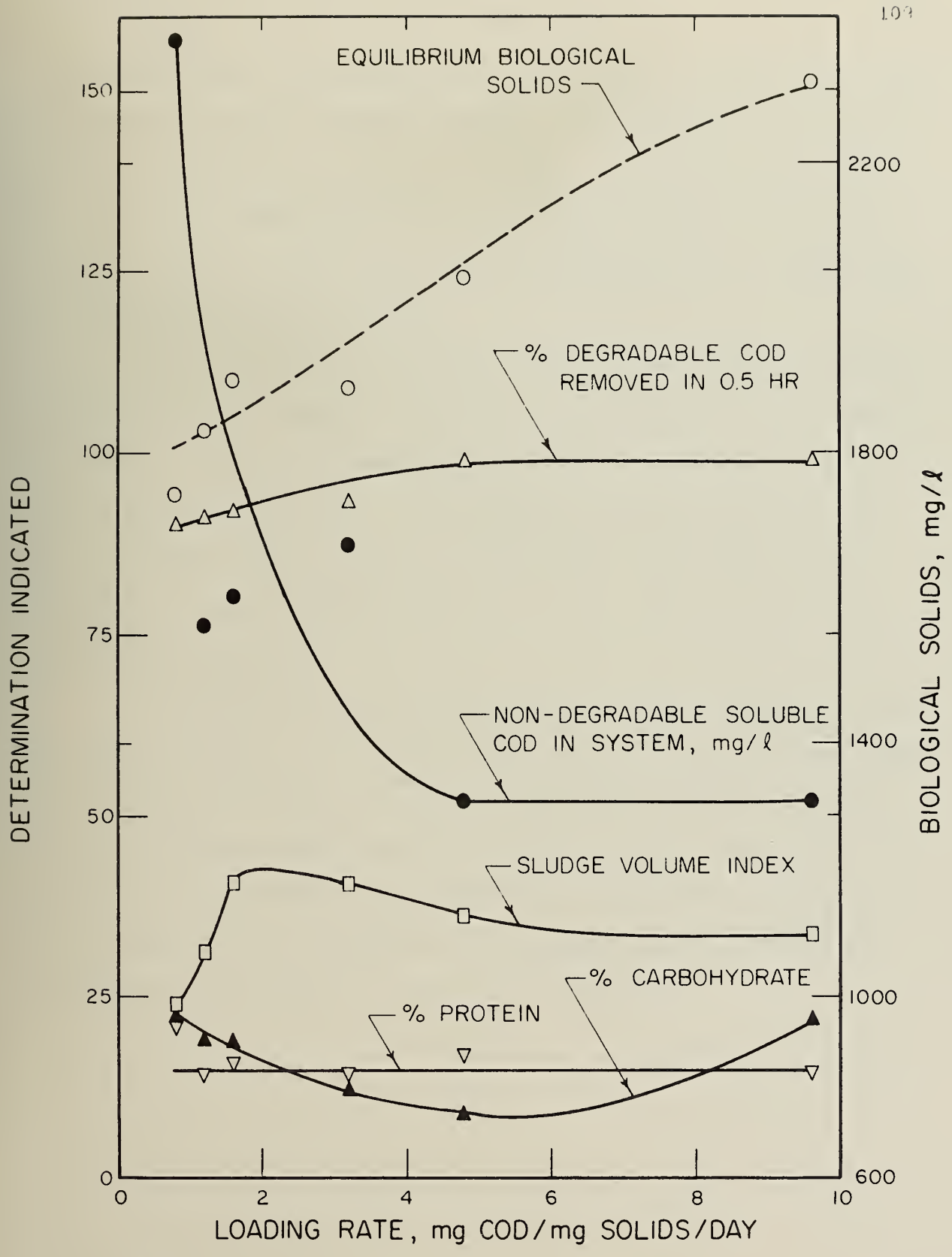

FIGURE 47 EFFECT OF LOADING RATE ON PERFORMANCE OF LABORATORY ACTIVATED SLUDGE UNIT

stabilization period was increased from 1 to 3 hours, as shown by the batch experiments described in previous sections. This increase in activity was probably an artifact of the batch experiments. The sludges used in these experiments were stabilized for 10 hours (Figure 9) prior to the initial contact. Therefore, the sludges were not in an equilibrium condition with the shorter stabilization periods.

The protein and carbohydrate contents of the sludge were calculated from the last observations in Figures 43 through 46. These values are plotted in Figure 47. Apparently the protein content of the sludge did not change significantly. The carbohydrate content first decreased with increasing loading rate and then increased at the highest loading. There does not seem to be any correlation of this observation with the performance of the unit.

In summary, the settling quality of the sludge appears to be the main design criteria for the stabilization period. For the substrate used in the present experiment a F/M ratio of 9.6 did not result in a bulking sludge. It was also found that stabilization of sludge impairs the ability of the organisms to remove substrate. Furthermore, long stabilization periods result in leaching of nondegradable soluble COD into the system and thus may lower the quality of the effluent.

VI. SUMMARY AND CONCLUSIONS

It has been shown that the primary mechanism in the removal of soluble substrates in solution, by a heterogeneous population of organisms characteristic of activated sludge, involves enzyme catalysed reactions. The primary removal rate, using glucose and α-methyl glucoside as substrates, could be saturated by increasing the F/M ratio. With α-methyl glucoside as substrate, the observed rate of removal was found to follow a theoretical enzymatic model rather than one associated with adsorption. The response of the primary rate of removal to a change in temperature also followed the pattern of biological reactions.

The above observations were in contrast with the widely accepted concept of adsorption as the primary mechanism for the removal of substrate in the contact stabilization process. Therefore, the existing formulations, which state that the rate of uptake is directly proportional to the substrate concentration, tend to make a liberal estimate of the capacity of the sludge to remove soluble substrate. A reduction in the efficiency of the contact stabilization process may be expected when it is overloaded with soluble wastes.

Using lactose as substrate and a lactose acclimated sludge, it was shown that starvation was not a sufficiently rigorous selecting agent to exclude organisms with inducible enzyme systems. Furthermore, the induction of β-galactosidase activity, which is an inducible system necessary for lactose metabolism, occurred too rapidly to allow time for the selective process to operate.

In the above system it was also shown that the β-galactoside

permease was the rate limiting enzyme. However, with extended stabilization periods the respiratory and synthesizing enzymes could have become rate limiting. These enzymes were retained by the organisms, in the absence of exogenous substrate, for a longer period of time as compared to the β-galactoside permease and β-galactosidase.

Both β-galactoside permease and β-galactosidase were shown to be inducible systems. Presence of thiomethyl-β-galactoside had a stabilizing effect on the activity of these enzymes during endogenous metabolism.

In batch experiments after the initial contact with the substrate, the sludge showed an increase in its capacity to remove substrate during the first few hours of stabilization. This was accompanied by a rise in the protein content of the sludge and a decrease in the intracellular concentration of intermediate products of metabolism. The increase in the protein content was attributed to the synthesis of new enzyme proteins, from the endogenous reserve, belonging to the respiratory and synthesizing systems. The increased sludge activity was, therefore, probably due to both synthesis of enzymes and decrease in the concentration of the intermediates.

The effect of different stabilization periods on the performance of a simulated contact stabilization unit was studied. A decrease in the stabilization time from nearly 11.5 to 0.5 hours resulted in an increase in the efficiency of the system. This observation suggested that the inactivation and subsequent synthesis of enzymes was the main reason for the initial increase in the activity of sludge in batch studies. The system was loaded to a maximum of 9.6 mg COD/mg sludge solids/day without resulting in a bulking sludge. This loading rate was nearly twenty times the recommended value for domestic waste. At high loading rates there was

also a decrease in the soluble COD of the effluent. The change in protein and carbohydrate contents of the sludge did not show any significant correlation with the performance of the system.

These observations suggest that the present design criteria of loading rates for domestic wastes should be used with caution with industrial wastes of different characteristics. A primarily carbohydrate waste, balanced with other growth requirements, can be treated at much higher loading rates than currently recommended for domestic wastes. Furthermore, the initial increase in activity of sludge during stabilization, in batch studies, should not be taken as the basis for selecting minimum stabilization time. Stabilization time should be chosen on the basis of the settling quality of sludge.

Based on these findings the following conclusions may be drawn:

(i) The primary removal of soluble substrates in a biological waste treatment process involves an enzyme catalyzed reaction.

(ii) The primary rate of removal of substrate can be saturated by increasing the F/M ratio.

(iii) Periodic starvation is not a sufficiently strong selecting agent to exclude organisms which have inducible enzyme systems.

(iv) The loss of substrate removal capacity of lactose acclimated sludge is due to the reduction in activity of the β-galactoside permease system.

(v) During long stabilization periods the respiratory and synthesizing enzymes also tend to lose their activity.

(vi) The initial increase in the activity of sludge organisms during stabilization, in batch studies, can be attributed to the synthesis of enzymes from endogenous reserve.

(vii) Batch studies should not be used to establish the minimum stabilization time in the treatment of a waste by contact stabilization process.

(viii) Settling quality of sludge appears to be the main criterion for selecting a stabilization period.

(ix) Depending upon the composition of the substrate, high loading rates can be adopted without impairing the settling quality of sludge.

While designing a contact stabilization system, therefore, the stabilization period should be kept to a minimum in order to utilize the full substrate removal capacity of the sludge. The time of stabilization should be chosen so as to give a loading rate which will not result in bulking sludge. To obtain this rate continuous flow pilot plant studies are necessary as batch studies tend to give misleading information. The contact time in the design will be governed by the degree of BOD removal desired. Under quantitative shock loads, which result in a saturating food to microorganism ratio, a fall in the BOD removal capacity of the plant should be expected. The situation may be minimized by increasing the stabilized return sludge.

VII. SUGGESTIONS FOR FUTURE WORK

In view of the conclusions arrived at from the present study, it is suggested that this investigation should be extended to include the following:

(i) Study of initial removal of different soluble substrates, both pure and heterogeneous in nature, and establishment of a substrate removal relationship.

(ii) Redefining of the loading and stabilization criteria of a contact stabilization process based upon the nature of waste and settling quality of the sludge.

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